SAUNDERS' INTERNATIONAL STUDENT EDITION

NINETEENTH EDITION

TEXTBOOK OF MICROBIOLOGY

Ву

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W. B. SAUNDERS COMPANY PHILADELPHIA LONDON TORONTO 1968

TOPPAN COMPANY, LIMITED TOKYO, JAPAN

268440

5760

Textbook of Microbiology

Original English Language Edition published by W. B. SAUNDERS COMPANY, Philadelphia and London, Copyright:

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Printed by Toppan Printing Company Limited, Tokyo, Japan

PREFACE

Microbiology continues to become more and more obviously a basic discipline, and one which underlies much of current biological thinking. Modern genetics and molecular biology, for example, are microbial genetics and molecular microbiology in present substance, and provide a foundation upon which concepts in these areas are developing. In this rapidly expanding role microbiology tends to become more and more complex, necessitating frequent revision of a book such as this.

The present volume has been extensively revised over its immediate predecessor. with changes and additions ranging from complete rewriting at the chapter level, through reorganization of a considerable portion of the subject matter, to new and completely rewritten sections. The chapter on medical mycology is completely new. with new illustrations. With more or less general acceptance of viral characteristics, largely structural in nature, a provisional classification of these agents has evolved, together with a number of contracted, yet still familiar names, which promises the evolution of an orderly relationship in this area of microbiology. While it is hardly practical at this time to accept the detailed differentiation and nomenclature which has been proposed by some, it is now possible to relate these viral agents to one another on a basis other than the doubtful one of host or tissue specificity. The viruses have, therefore, been reorganized, both in terms of their taxonomy and in the order of their consideration. The latter is reflected in the transposition of the discussions of a number of them, such as measles and rabies viruses, and in the reorganization of the arboviruses. It is possible, too, that the application of the methods of numerical taxonomy, discussed in a new section, to the bacteria may lead to a more realistic appraisal of the interrelationships of these, and possibly eventually other, microorganisms.

The spread of cholera within the past few vears, consistent with the initial stages of a new pandemic, has stimulated a great deal of research on this disease, leading to the elucidation of certain of the mechanisms of its pathogenesis and pathophysiology and to a broader concept of the causative organism as a group of biotypes. Such accumulation of new information has required a large amount of rewriting of the chapter devoted to these microorganisms. Presently available knowledge of the function of ribosomes and the functional types of RNA has led to the complete rewriting of relevant sections, as has the eludication of bacterial nuclear structure and function, including such peripheral matters as the episomal aspects of drug resistance among the gram-negative enteric bacilli. Elegant studies on the pathogenesis of bacillary dysentery have led to an understanding of the pathogenesis of these infections and certain genetic aspects of microbial virulence.

Similarly, very great progress has been made in the study of antibody as immunoglobulins, though it is as yet too soon to relate with certainty some of these, such as IgA secretory immunoglobulin, to effective immunity. The relation of ontogeny to the immune response and the role of the thymus as a source of antibody-forming cells are now beginning to emerge to form the basis of new sections. The material on the measles and rubella viruses is entirely new, and the diverse etiology of the common cold has become increasingly evident, lead-

PREFACE

ing to an entirely rewritten and expanded section. The section on food poisoning and foodborne infection has been consolidated from its dispersal among the various causative microorganisms and appears as a completely rewritten and considerably expanded section, including consideration of control of such disease.

The inclusion of bibliographies at the end of each chapter, initiated in the immediately preceding edition, has been continued, with various research papers, reviews, and monographs being given in full, including titles in the original language. Inevitably any such compilation is individualistic in nature and undoubtedly omits publications which should have been included. The author has reason to believe, however, that such bibliographies, current and updated, have been useful to their original purpose -i.e., to provide the student with an entrée to the rich and rewarding literature of microbiology-and hopes that they will continue to fulfill this intended function.

Dr. J. W. Moulder has been good enough

to continue his complete responsibility for consideration of microbial metabolism, and Dr. R. M. Lewert has prepared the revision of the chapter on medical parasitology. The author is especially indebted to Dr. J. W. Rippon for his extensive work, including the preparation of very many original illustrations and the complete rewriting of the chapter on medical mycology. The author is grateful also to many other co-workers and friends for advice, criticism, and access to unpublished material, especially Dr. R. S. Benham and Miss Isabelle Havens, Dr. E. H. LaBrec, and Dr. S. B. Formal. Mrs. Barbara Hilbrant has provided invaluable secretarial assistance. And to more than any other one individual, the author is indebted to his wife, not only for her forebearance during some thirty years of association with this book, but also for her assumption of complete responsibility for all bibliographic matters.

WILLIAM BURROWS

CONTENTS

Chapter 1	
THE HISTORICAL DEVELOPMENT OF MICROBIOLOGY	1
Microscopy	
Spontaneous Generation	
Fermentation and Biochemical Physiology	
Infectious Disease	
Viruses	
Immunity	
Chemotherapy	
Microbial Genetics	
Reconsideration	11
Chapter 2	
Laboratory Methods	13
Sterilization	13
Preparation of Culture Mediums	
Microscopic Examination	
Motility	
Staining	
Culture of Bacteria	
Systematic Study of Bacteria in Pure Culture	23
Animal Inoculation	24
Propagation of Viruses and Rickettsiae	25
The Embryonated Egg	25
Tissue Culture	
Immunological Methods	29
Chapter 3	
THE PHYSICAL AND CHEMICAL STRUCTURE OF	
MICROORGANISMS	
Microscopy	
The Morphology of Bacteria	39
The Bacterial Cell	39
The Structure of Bacterial Cells	43

External Structures	43
Staining Reactions	47
The Cell Wall and Plasma Membrane	49
Internal Structures	54
Intracellular Localization of Enzymes	61
Colonial Morphology of Bacteria	62 64
The Morphology of Rickettsiae and Viruses The Morphology of Rickettsiae	64
The Psittacosis—Lymphogranuloma Venereum	04
Organisms	66
The Morphology of Viruses	67
The Bacterial Viruses (Bacteriophages)	69
Animal Viruses	72
The Insect Viruses	75
Plant Viruses	76
Comparative Morphology of Microorganisms	78
Chapter 4	
THE GROWTH OF MICROORGANISMS	81
The Growth of Bacteria	81
The Growth of Bacterial Populations	82
The Replication of Viruses	86
The Replication of Bacteriophage	87
The Replication of Animal Viruses	92
Multiple Infections and Interference	99
The Comparative Physiology of Growth	101
Chapter 5	
BACTERIAL METABOLISM	105
	103
by James W. Moulder, Ph.D.	
Respiration	
Some Basic Concepts of Respiration	106
Respiratory Enzymes in Bacteria	
The Relation of Bacteria to Molecular Oxygen	111
Conservation and Transfer of the Engery Released	110
in Biological Oxidations	
Carbohydrate Metabolism	
Breakdown of Hexoses and Pentoses	
Breakdown of Pyruvic Acid	
The Tricarboxylic Acid Cycle	
Fixation of Carbon Dioxide	131
Bacterial Fermentations	
Lipid Metabolism	138
Inorganic Nitrogen Metabolism	140
Metabolism of Amino Acids and Proteins	142
Breakdown of Proteins	142
Breakdown of Amino Acids	
Synthesis of Amino Acids	145
Metabolism of Nucleic Acids, Nucleotides, and Related Substances	
	150
Breakdown of Nucleic Acids	
Breakdown of Nucleic Acids	159

CONTENTS	· •	/ii

Regulation of the Biosynthesis of Purines and	
Pyrimidines	163
Synthesis of Nucleic Acids and Proteins	162
Nutrition of Bacteria	160
Nutrition of Bacteria	100
Chapter 6	
PHYSICAL AGENTS, BACTERICIDAL SUBSTANCES	400
(Disinfectants), and Chemotherapeutic Drugs	
Physical Agents	178
Disinfectants	
The Chemotherapeutic Drugs	. 197
Synthetic Compounds	. 198
The Antimetabolite Theory of Antibacterial	
Activity	
Antibiotic Substances	
The Application of Chemotherapeutic Agents	210
Chapter 7	
MICROBIAL VARIATION AND GENETICS	. 218
The "Unique" Features of Microorganisms	. 218
Observed Variations of Microorganisms	
Morphological Variation	
Physiological Variation	
Environmental Selection	
Adaptation	
Attenuation	
Biochemical Variation	
Resistance to Chemotherapeutic Agents	. 231
Adaptive Enzymes	
Conjugation and Recombination	. 238
Transduction (Transfection)	. 240
Transformation	. 241
The Nature of Microbial Variations	. 243
Mutation	
Induced Adaptation	
Microbial Phylogeny	. 247
Chapter 8	
THE TAXONOMY OF MICROORGANISMS	. 252
Ch 0	
Chapter 9	
THE PATHOGENIC MICROORGANISMS AND DISEASE	. 259
The Specific Microbial Etiology of Infectious Disease	
Microbial Virulence	
Microbial Toxins	
Exotoxins	
Endotoxins	
Other Toxic Substances	
Capsules	
Miscellaneous Factors	
Toxicity of Host Origin	
Resistance	277

viii CONTENTS

Chapter 10	
THE EPIDEMIOLOGY OF INFECTIOUS DISEASE	293
Epidemiological Types of Infectious Disease	295
The Microbial Population	297
The Host Population	300
Experimental Epidemiology	
Epidemiological Data and Their Interpretation	305
The Control of Infectious Disease	307
Chapter 11	
The Microbiology of Water and Sewage	
Water	310
Sewage	319
Chapter 12	
The Microbiology of Milk and Food	322
Milk	
Food Poisoning and Foodborne Infection	
Food Poisons of Bacterial Origin Foodborne Bacterial Infections	329
Foodborne Parasitic Infections	
Control of Foodborne Disease	
Chapter 13	
IMMUNITY: ANTIGENS, ANTIBODIES, AND THE ANTIGEN-	
ANTIBODY REACTION	335
Antigens	335
Properties of Antigens	
Antigenic Specificity	
The Chemical Basis of Specificity The Antigenic Structure of Microorganisms	
Antibodies	
The Antigen-Antibody Reaction	
Chapter 14	
IMMUNITY: THE SEROLOGICAL REACTIONS	364
Precipitins	364
Agglutinins	367
Immunofluorescent Staining	
AntitoxinsLysins	
Opsonins	
Protective and Neutralizing Antibodies	
가장 보는 보고 그는 그래, 나를 내용하고 있을까 살을 보고 말하는 걸다.	
Chapter 15	
THE IMMUNE STATE	
Humoral Immunity	388
Cellular Immunity	

CONTENTS	ix
Natural Immunity Acquired Immunity Active Immunity	399
Passive Immunity Hypersensitivity	403
Anaphylaxis	404
Allergy and Atopy Hypersensitivity in Infection. The Relationship of Hypersensitivity and	408
Immunity	412
Chapter 16	
The Staphylococci	415
Other Micrococci	
Chapter 17	
The Streptococci	430
Streptococcal Infection of the Skin and	
Subcutaneous Tissues Streptococcal Infection of the Upper Respiratory	
Tract	
Rheumatic Fever	447
Acute Glomerulonephritis	449
Chapter 18	
THE PNEUMOCOCCI	. 453
Chapter 19	
THE GRAM-NEGATIVE PATHOGENIC COCCI (NEISSERIA):	
THE GONOCOCCUS AND THE MENINGOCOCCUS	
The Meningococcus	. 470
Other Gram-negative Diplococci	. 476
Chapter 20	
THE ENTERIC BACILLI: THE COLIFORM BACTERIA,	
PRIEDLÄNDER'S BACILLUS (PNEUMOBACILLUS), AND	470

CONTENTS

Classification of Enteric Bacilli The Coliform Bacilli Physiological Differentiation of Coliform Bacilli The Immunological Relationships of the Coliform	483
Bacilli	487 489
Proteus	490
Chapter 21	
THE ENTERIC BACILLI: THE SALMONELLA GROUP	495
Salmonella Infections Salmonella Gastroenteritis Typhoid and Paratyphoid Fevers Alcaligenes faecalis	504 505
Chapter 22	
THE ENTERIC BACILLI: THE DYSENTERY BACILLI	516
Shigella dysenteriae (Group A)	
Shigella flexneri (Group B)	
Shigella sonnei (Group D)	521
Bacillary Dysentery	
Chapter 23	
THE CHOLERA VIBRIO AND RELATED FORMS	530
Chapter 24	
BRUCELLA: UNDULANT FEVER; CONTAGIOUS ABORTION OF CATTLE	5 47
Brucella bronchiseptica	
bracena oronemseptica	333
Chapter 25	
PASTEURELLA AND ACTINOBACILLUS: HEMORRHAGIC SEPTICEMIA; PLAGUE; TULAREMIA; GLANDERS;	
ACTINOBACILLOSIS	557
Pasteurella	557
Pasteurella pestis – The Plague Bacillus Pasteurella pseudotuberculosis	558
Pasteurella tularensis	567
Actinobacillus	570
The Glanders Bacillus (Actinobacillus mallei) Whitmore's Bacillus	
Actinobacillosis	

CONTENTS			Χĺ

Chapter 26	
THE HEMOPHILIC AND RELATED BACTERIA	578
Hemophilus influenzae (Pfeiffer's Bacillus)	578
The Koch-Weeks Bacillus	
Hemophilus pertussis (Bordetella pertussis)	582
The Morax-Axenfeld Diplobacillus (Hemophilus	
duplex)	586
Ducrey's Bacillus (Hemophilus ducreyi)	386
Chapter 27	
PSEUDOMONAS; LACTOBACILLUS; LISTERIA; ERYSIPELOTHRIX;	
BACTEROIDES; STREPTOBACILLUS; BARTONELLA;	
Mycoplasma; Donovania	
Pseudomonas aeruginosa (Pyocyanea)	589
Lactobacillus	
Related Bacteria	
Listeria monocytogenes Erysipelothrix rhusiopathiae	
Nonspore-forming Anaerobic Bacilli (Bacteroides)	599
Streptobacillus moniliformis (Rat-Bite Fever, Haverhill	
Fever)	
Bartonella	603
Mycoplasma – The Pleuropneumonia-like Organisms	605
(PPLO) Mycoplasma of Man	
Donovania granulomatis.	
Chapter 28	
	(11
Bacillus—The Spore-Forming Aerobes	
Related Bacilli	
Rotated Basini	020
Charten 20	
Chapter 29	
CLOSTRIDIUM—THE SPORE-FORMING ANAEROBES	
Clostridium Tetani	
Gaseous Gangrene	
The Vibrion Septique, Clostridium septicum Clostridium welchii (Clostridium perfringens)	
Clostridium novyi (Clostridium oedematiens)	
Clostridium histolyticum	
Clostridium sporogenes	. 640
Clostridium chauvoei (Clostridium feseri)	. 641
Clostridium botulinum	. 642
Chapter 30	
CORYNEBACTERIUM (THE DIPHTHERIA BACILLUS)	. 649
The Diphtheroid Racilli	660

Chapter 31	
Mycobacterium	663
The Tubercle Bacilli	
Mycobacterium leprae (Hansen's Bacillus)	678
Mycobacterium leprae murium	682
Other Acid-fast Bacilli	682
Chapter 32	
MEDICAL MYCOLOGY: THE PATHOGENIC FUNGI AND THE	
PATHOGENIC ACTINOMYCETES	687
by John W. Rippon, Ph.D.	
The Pathogenic Actinomycetes	600
Actinomycosis	691
Nocardiosis	695
Other Actinomycetous Infections	697
Mycetoma (Maduromycosis)	
The Pathogenic Fungi.	
The Superficial Mycoses	
Tinea Versicolor (Pityriasis Versicolor)	
Tinea Nigra	
Piedra	
The Cutaneous Infections (Dermatophytoses)	
The Pathogenic Yeasts	
Candidiasis	
The Subcutaneous Mycoses	
Chromoblastomycosis	
Sporotrichum schenckii (Sporotrichosis)	718
Subcutaneous Phycomycosis	
The Systemic Mycoses	
The Dimorphic Systemic Fungi	
North American Blastomycosis	
Coccidioidomycosis	
Rhinosporidiosis	
Adiospiromycosis	
Paracoccidioidomycosis	
Histoplasmosis	
Other Systemic Mycoses	
Cryptococcosis	733
Aspergillosis	
Mucormycosis	727
Mycotoxicosis	
	730
Chapter 33	
THE SPIROCHETES	745
The Spirochetes of the Relapsing Fevers (Borrelia)	
Spirochetes of the Mouth	750
Treponema and the Treponematoses	
Treponema pallidum (Syphilis)	
Nonvenereal Syphilis	757
Treponema pertenue (Yaws)	757
Treponema carateum (Pinta)	759

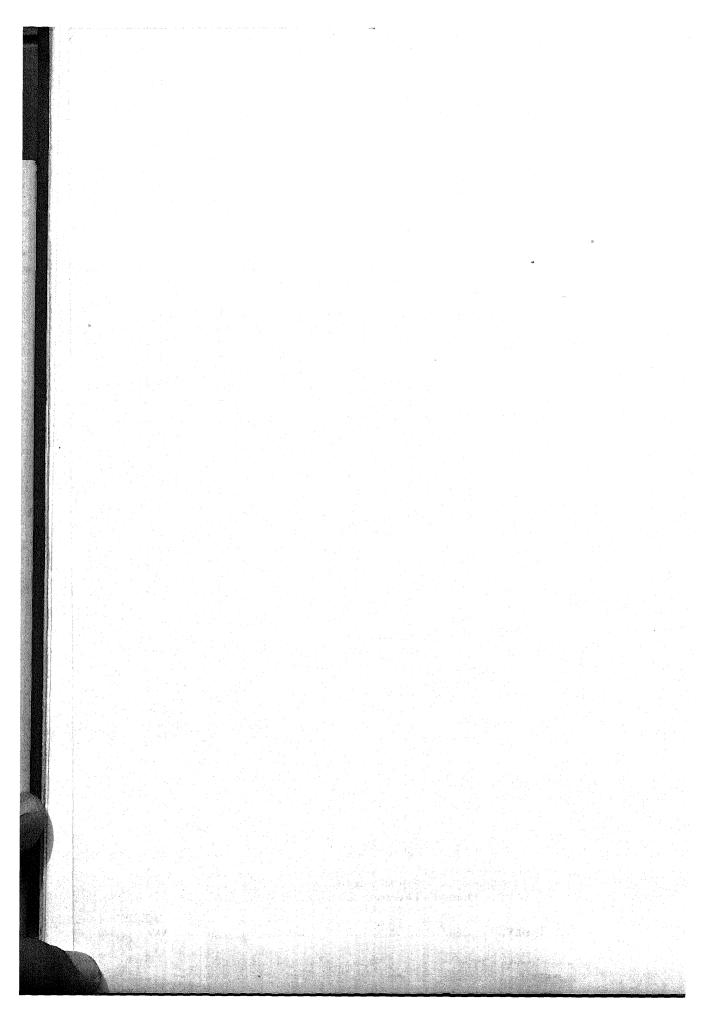
CONTENTS	xiii
Leptospira and Leptospirosis	
Weil's Disease (Infectious Jaundice)	
Canine Leptospirosis	
Swamp FeverLeptospiral Infections of the Far East	764
Saprophytic Leptospira	
Rat-Bite Fever (Spirillum morsus muris)	765
Chapter 34	
Medical Parasitology	769
by Robert M. Lewert, Sc.D.	
The Protozoa	770
The Intestinal Amebae (Sarcodina)	
Ciliophora	
Intestinal Flagellates (Mastigophora)	
Hemoflagellates (Mastigophora) The Trypanosomes	776
The Leishmanias	
Sporozoa	
Intestinal Sporozoans	791
Parasites of Uncertain Affinities	
The Metazoa	
Platyhelminthes	
Cestoda	
Nematoda	
The Filaria	818
Chapter 35	
Rickettsia	825
The Typhus Fevers	
Epidemic Louseborne Typhus Fever	
Murine Typhus	
Immunization	
The Spotted Fevers	
Related Rickettsial Infections	
Tsutsugamushi Disease (Scrub Typhus, Mite Typhus)	
Q Fever	
Trench Fever	842
Laboratory Diagnosis of Rickettsial Disease	843
Chapter 36	
THE PSITTACOSIS – LYMPHOGRANULOMA VENEREUM GROUP OF MICROORGANISMS	846
Psittacosis and Ornithosis	
Lymphogranuloma Venereum	
Trachoma and Inclusion Conjunctivitis	
Trachoma	855
Inclusion Conjunctivitis	856

Chapter 37

	Chapter 37	
	VIRUSES OF EXANTHEMATOUS DISEASE AND THE TUMOR	
Vir	USES	
	The Pox Group of Viruses	859
	Variola and Vaccinia	862
	Pox Diseases of Lower Animals	
	The Herpesviruses	867
	Herpes Simplex	
	Herpetic Infections of Lower Animals	
	Pseudorabies	
	B VirusVirus III	
	Virus IIIVirus IIIVirus III	
	Cat-scratch Fever	
	Infectious Mononucleosis	
	Salivary Gland Disease Viruses	
	Tumor Viruses	
	Molluscum Contagiosum	875
	Myxoma and Fibroma Viruses	
	Infectious Wart Virus	
	Rabbit Papilloma Virus	877
	Vacuolating Virus	
	Viral Agents of Mouse Neoplastic Disease	
	Viruses of Avian Neoplastic Disease	880
	Chapter 38	
Гн	MYXOVIRUS GROUP (INFLUENZA, MUMPS, MEASLES,	
Rui	BELLA, RABIES) AND RELATED VIRUSES	885
	The Myxoviruses	885
	The Influenza Viruses	
	The Influenza-like and Other Respiratory Viruses	893
	Parainfluenza Viruses	
	Respiratory Syncytial Virus (RS Agent)	
	The Mumps and Related Viruses	
	Mumps	
	Newcastle Disease Virus	
	The Measles (Rubeola) Virus	898
	The Rubella Viruses	
	The Rabies Virus	903
	Chapter 39	
Тні	E PICORNAVIRUSES (POLIOVIRUSES, COXSACKIE VIRUSES,	
	HO VIRUSES, COMMON COLD VIRUSES); THE HEPATITIS	
VIR	uses; The Adenoviruses	
	The Polioviruses	
	Encephalomyelitis of Mice	
	Teschen Virus (Porcine Encephalomyelitis)	
	The Coxsackie Viruses	
	The Enteric Orphan Viruses	
	ECHO Viruses	
	Reoviruses	926
	Enteric Orphan Viruses of Lower Animals	927
	The Encephalomyocarditis Viruses	720

CONTENTS		XV
The Common Cold Viruses		
Foot-and-Mouth Disease		930
Viral Diarrheas	*******	931
Viral Hepatitis		
Infectious Hepatitis		
Serum Hepatitis		
Hepatitis of Lower Animals		
The Adenoviruses		
Chapter 40		
Arboviruses (Yellow Fever, Mose ephalitides, Tickborne Encephalit		

Infectious Hepatitis	932
Serum Hepatitis	
Hepatitis of Lower Animals	
The Adenoviruses	
Chapter 40	
THE ARBOVIRUSES (YELLOW FEVER, MOSQUITO-BORNE	
Encephalitides, Tickborne Encephalitides, Dengue);	
The Lymphocytic Choriomeningitis Virus	942
The Encephalitis and Related Viruses of Group A	943
Equine Encephalitis Viruses	943
Western Equine Encephalitis	944
Eastern Equine Encephalitis	
Venezuelan Equine Encephalitis	947
Sindbis Virus	948
Chikungunya Virus	948
Semliki-Mayaro Virus	
Semliki Forest Virus	
Mayaro Virus	
O'Nyong-Nyong Virus	
Mosquito-borne Viruses of Group B	
St. Louis Encephalitis	
Japanese B Encephalitis	
Murray Valley Encephalitis	
West Nile Virus	
Dengue	
Ntaya Virus	
Uganda S Virus	
Zika Virus	
Ilheus Virus	
Yellow Fever	
The Tickborne Viruses of Group B	
Russian Spring-Summer Encephalitis	
Louping Ill	962
Kyasanur Forest Disease	962
Powassan Virus	
Colorado Tick Fever	
The Bunyamwera Group of Viruses	
Bunyamwera Virus	
Cache Valley Virus	065
Other Mosquito-borne Viruses	
Sandfly Fever	
Rift Valley Fever	
California Virus	
Turlock Virus	
Bwamba Fever Virus	
Interrelationships Among Arboviruses	
Lymphocytic Choriomeningitis	
Durand's Disease	
	211



Chapter One

THE HISTORICAL DEVELOPMENT OF MICROBIOLOGY

The term microbiology is commonly used in a more narrow sense than its etymology suggests. It does not refer to the broader subject of small or minute living organisms, but rather has come to be restricted to those microorganisms that are directly or closely related to human activity and welfare.

The microorganisms as a group resist precise taxonomic limitation in that they spread over both the plant and animal kingdoms, from the fungi of obvious plant affinities to the unicellular and small multicellular animals. In this scheme of things the true bacteria, or Eubacteriales, occupy a position between the two kingdoms and in a real sense constitute a link between them. The viruses, distinguished by their obligate parasitic relationship to the cells of their hosts, are perhaps to be regarded as an offshoot of the bacteria, and are of unique interest in that many of them seem to occupy a borderline position between the living and the nonliving.

The consequences of the varied activities of many of the microorganisms have been familiar to man from prehistoric times. Decomposition of organic matter and especially spoilage of foods, acetic and lactic acid fermentations and alcoholic fermentation, degradation of proteins with the production of new and desirable flavors in certain foods, and the occurrence of infectious disease are obvious examples of familiar phenomena now known to be of microbial etiology.

The existence of these etiological agents may be inferred from such consequences of their activity, and their living nature from the ability to reproduce the observed effects indefinitely in series by, for example, the transfer of a small portion of a fermenting mixture to fresh, unfermented substrate. It is probable that such inferences were made early, for many statements may be found in both ancient and later writings which may be interpreted as supporting this idea. Thus Lucretius writes of "the seeds of disease" in his *De Rerum Natura*, Fracastorius of Verona in 1546 suggested a contagium vivum as a cause of disease, and von Plenciz accounted for the specificity of disease on the basis of a microbial etiology in 1762.

Nevertheless, appreciation of the abstract concepts commonplace in modern science is a relatively recent development, and even yet these are often accepted by man in general more on faith than on intellectual understanding. So an essential feature of the beginnings of a scientific microbiology was the demonstration of these agents in such a way that they were more or less directly evident to the senses. This was accomplished by the development of optical systems of sufficient precision to allow them to be seen.

MICROSCOPY

The name of Antony van Leeuwenhoek is inseparably associated with the early development of microscopy. Van Leeuwenhoek lived in the latter part of the seventeenth and early part of the eighteenth centuries (1632–1723) in Delft. He held a political sinecure and was able to devote the greater part of his time to the hobby of lens grinding. He not only made the best lenses available to that time, but used them in examination of a wide variety of materials that interested him. It was characteristic that he first observed bacteria in an attempt to discern in visual

terms the nature of the taste of pepper. There is no doubt that he actually observed these microorganisms for he made recognizable drawings of them that were reproduced in his report⁹ of his observations to the Royal Society. In the relevant portion of this report he wrote:

Having several times endeavoured to discover the cause of the pungency of pepper upon our tongue, and the rather because it hath been found, that though pepper had layn a whole year in vinegar, yet it retained still its pungency; I did put about 1/3 of an ounce of whole pepper in water, placing it in my study, with this design, that the pepper thereby being rendered soft, I might be enabled the better to observe what I had proposed to myself. This pepper having lain about three weeks in the water, to which I had added some snow water, the other water being in great part exhaled. I looked upon it the 24th of April, 1676 and discern'd in it, to my great wonder, an incredible number of little animals, of divers kinds. . . . The 4th sort of creatures, which moved through the three former sorts, were incredibly small and so small in my eye, that I judge, that if 100 of them lay one by another, they would not equal the length of a grain of coarse sand; and according to this estimate, ten hundred thousand of them could not equal the dimensions of a grain of such sand.

A few of the hundreds of microscopes he constructed are still available, and it is evident that the maximum magnification he reached was approximately 300 diameters. This is not sufficient to allow observation

unquam sanguinem emittat. Nec tan sunt puri, quin, ubi eos per specult tuerer, viderim crescentem inter de

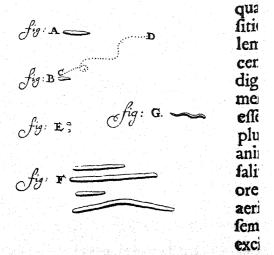


Figure 1. The first pictorial representation of bacteria. (Reproduced from Arcana Naturae delecta ab Antonio van Leeuwenhoek. Delphis Batavorum apud Henricum Crooneveld. 1695.)

of objects as small as bacteria by transmitted light. While van Leeuwenhoek was never willing to reveal his method of illumination, it seems probable that he used reflected light as it is used in the modern darkfield microscope.

The direct demonstration of living organisms of such small dimensions was a notable achievement, but any relation of such forms to natural phenomena such as fermentation or infectious disease either escaped van Leeuwenhoek or was of no interest to him. Their possible relation to gross phenomena was apparent to at least some, and possibly many, scientific men of his day. For instance, in a comment on a report of an epidemic disease of cattle published five years later, his contemporary, Slare, wrote:¹⁰

I wish Mr. Leeuwenhoek had been present at some of the dissections of these infected Animals, I am perswaded he would have discovered some strange Insect or other in them.

But systematic study was delayed for many years, and it was nearly a century later. in 1786, that the Danish zoologist, O. F. Müller, studied the bacteria and succeeded in discovering many details of their structure. He left drawings so accurate that the bacteria he showed can be identified today as belonging to one or another of the chief divisions. Somewhat later, in 1838, Ehrenberg published his work Infusionstierchen in which he put the study of these microorganisms on a systematic basis and was able to establish a number of groups by clearly recognizing fundamental morphological distinctions, such as those differentiating the spirochetes from certain of the protozoa with which others had grouped them. Some of the names he used, such as "bacterium" and "spirillum," are still current in bacteriological nomenclature though with somewhat changed significance. In the next two or three decades considerable knowledge was amassed by such men as Dujardin, Perty, Nägeli, Cohn, and others. It is of more than passing interest that Cohn was Koch's professor.

The perfection of the modern compound microscope, with achromatic and then with apochromatic and fluorite objective lenses, markedly facilitated the study of the morphology of microorganisms, and at least certain of the gross internal structures of the bacterial cell as well as some of the larger viruses could be observed. This made possible accurate differentiation of microorganisms on a morphological basis and provided the basic criteria for characterization of the larger forms, the fungi, protozoa, and metazoa. The introduction of phase miscroscopy in the 1940's further facilitated certain aspects of microscopy, especially the observation of microorganisms in the living state, by accentuation of slight differences in the refractive indices of intracellular elements.

Optical microscopy is, nevertheless, limited by the resolution that may be obtained with visible light to a working limit in magnification of 1000 to 2000 diameters and resolution of objects as small as 0.2μ . It was obvious that such resolution limits are inadequate for study of much of the intracellular structure of bacteria and, further, that living organisms beyond the limits of optical resolution existed. The darkfield microscope, although facilitating the observation of tenuous microorganisms such as the spirochetes and structures such as flagella, shows minute objects only as points of light against a dark background without increasing resolution. The use of ultraviolet light in substantially the conventional compound microscope equipped with quartz lenses resulted in some small increase in resolving power in the 1920's, but the fundamental limitation of the size of the object in relation to the wave length of the light used still remained.

Within the next decade or two the electron microscope was developed in practical usable form. It constituted an important advance in microscopy in that an object or structure casting a "shadow" in the electron beam may be resolved, and working magnifications of 30,000 diameters with excellent resolution were obtained. Thus it became possible to photograph microorganisms beyond the limits of optical resolution and to demonstrate directly morphological characteristics down to and including the orientation of the macromolecules that represent the complete unit of certain of these agents.

SPONTANEOUS GENERATION

For many years it was generally believed that living organisms could arise *de novo* and fully formed from decomposing organic matter. The development of snakes from

horse hairs standing in stagnant water and the appearance of mice in decomposing fodder are familiar examples that, in fact, persist in some areas to the present time. The fallacies in such beliefs were suspected by some, and in the seventeenth century a number of individuals carried out experiments designed to show whether living organisms had their origin only in other living organisms (biogenesis), or appeared spontaneously in decomposing organic matter (abiogenesis).

The poet-physician Redi carried out experiments in the middle of the seventeenth century that showed, contrary to popular belief, that maggots were not formed spontaneously in decomposing meat but were fly larvae hatched from eggs deposited in the meat. Spallanzani, an Italian monk, showed further that putrescible meat infusions did not spoil when properly heated and did not contain living organisms even though kept over long periods. Needham, an Irish priest, took issue with Spallanzani on the basis of similar experiments in which spoilage took place and living organisms appeared in spite of previous heating. A second series of elaborate experiments by Spallanzani corroborated his earlier findings and indicated the fallacies in Needham's experiments. It was evident that the source of microorganisms was air, and this was convincingly demonstrated by many other workers, including Schulze, Schwann, Schröder and von Dusch, and Tyndall.

The whole question seemed settled conclusively in favor of biogenesis when it was raised again in the middle of the nineteenth century by the work of the eminent French chemist Pouchet. He made the same kind of technical errors as some of his predecessors had, so that his evidence supported the hypothesis of spontaneous generation of life. It was at this point that Pasteur entered the renewed controversy through his early studies on fermentation, and he was able to show beyond any reasonable doubt that the source of microorganisms was air by demonstration of the varying numbers of microorganisms in air from various sources, and by ingenious experiments in which the heated material had free access to air through tortuous passages that allowed bacterialaden dust to settle before the air reached the putrescible test materials.

The validity of biogenesis has not since

been seriously questioned. While biogenesis may seem self-evident today, it was a matter of fundamental importance to biology that it be fully established. Had this not been done the specific microbic etiology of fermentation, decay, infectious disease, and similar phenomena could not have been established. At the same time present concepts of evolution, and biochemical evidence in particular, seem to point inevitably toward an original emergence of life from the non-living (Chap. Three).¹¹

FERMENTATION AND BIOCHEMICAL PHYSIOLOGY

By the middle of the nineteenth century the general nature of organic material was becoming relatively clear, but the natural decomposition of these substances was not, in that the part played by microorganisms in the processes of putrefaction, decay, and fermentation was not known. This is something of an inconsistency in that in some of the earlier studies on spontaneous generation, the decomposition of meat infusions was taken as evidence of the presence of living organisms and, since the organisms were not found in the absence of decomposition, a causal relation was suggested.

The plant-like nature of yeast had been shown by Caignard-Latour and by Schwann, but the chemists of the day, Liebig, Berzelius, and Wöhler, regarded the presence of yeast cells in a fermenting mixture as no more than incidental to the decomposition which was considered to have a purely inanimate basis.

Pasteur (1822-1895), originally trained as a chemist, had done his early work on stereoisomerism. The formation of optically active amyl alcohol during the course of the lactic acid fermentation led him to the study of the processes of fermentation. Having first established the validity of biogenesis, it was not difficult to prove that fermentations resulted from the physiological activity of living, growing microorganisms. It is now clear, of course, that while the living organism is an indispensable adjunct to fermentation, catalysis of the process is a function of the enzymes formed by the living cell and may be brought about by cell-free preparations.

The specificity of fermentations, the fact that different kinds of fermentations characterized by differences in predominant end products result from the activities of different kinds of microorganisms, grew out of this work and was the beginning of the establishment of the important principle of specific microbic etiology. Pasteur applied this in his work on the "diseases" of beer and wine and showed these were none other than secondary fermentations brought about by extraneous microorganisms that gave undesirable end products. He controlled the fermentative process by gentle heating to destroy undesirable microorganisms, followed by inoculation of the fermentable mixture with microorganisms that brought about the desired fermentation. This method of gentle heating, now also widely applied to destroy pathogenic microorganisms that may be present in milk, has been named "pasteurization" in his honor.

Study of the mechanisms of fermentation was tremendously stimulated by the commercial value of end products such as ethanol, lactic and acetic acids, glycerol, butanol, and acetone on the one hand, and on the other by the obvious similarities in the metabolism of carbohydrate by many microorganisms and by higher animals, including man. The former consideration led to the establishing of a new industry concerned with the large scale production of organic solvents and subsequently other microbial products, especially vitamins and antibiotics.

The chemistry of microorganisms, necessitated by their morphological simplicity and physiological complexity, grew side by side with the chemical approach to mammalian physiology, eventually fusing into presentday biochemistry. As indicated above, the initial common ground was carbohydrate metabolism and the respiratory processes, astonishingly closely similar in such widely dissimilar organisms. Thus, the phenomenon of anaerobic respiration, first observed by Pasteur in fermenting mixtures and received with incredulity at the time, is now commonplace in biochemical physiology. More detailed study showed that the catalysis of the processes of respiration is substantially the same also, and characterization of the iron porphyrin and flavoprotein enzymes, for example, was markedly facilitated by their availability in microorganisms. Many of the vitamins required by mammals, especially those of the B group, are also required by microorganisms, and apparently function in the same manner, *i.e.*, as prosthetic groups or precursors of essential enzyme systems. Similarly, many microorganisms require preformed amino acids in much the same way that the mammal does.

In other respects the physiological potentiality of microorganisms goes far beyond that of any other living organisms. One gets the impression that these forms constituted a testing ground for a variety of physiological machinery, only some kinds of which have had sufficient survival value to persist on any large scale through the evolutionary process. While mammalian metabolism is simulated by some microorganisms, others are photosynthetic and resemble the green plants in the photochemical reduction of carbon dioxide or differ in that the photochemical reactions are coupled with the metabolism of inorganic sulfur compounds. Still others are chemoautotrophic in that they derive energy for the reduction of carbon dioxide from the oxidation of inorganic substrates such as hydrogen, or inorganic compounds of nitrogen, sulfur, iron, and manganese, and the nitrogen-fixing bacteria assimilate atmospheric nitrogen either alone or in symbiosis with leguminous plants.

Their sorting out by Winogradsky, Beijerinck, Hellriegel and Wilfarth, and others, beginning around the turn of the century. represents a remarkable achievement, not only in the isolation of physiological types of microorganisms for which there was no precedent, but also in the elucidation of the mechanisms of the cyclic transformations of the elements in nature and the microbial basis of soil fertility. Thus the autotrophic nitrifying bacteria, uniquely characterized by the oxidation of ammonia to nitrite, and of nitrite to nitrate, make possible the complete cycle of transformation of nitrogen compounds. The nitrogen leaking from the fixed form is more than replaced by microbial fixation, and the nature of the rejuvenation of depleted soil by cultivation of legume crops is made clear.

INFECTIOUS DISEASE

Sometimes new vistas are opened prematurely and not appreciated at the time, or too late and are anticlimactic, but occasionally new concepts coincide with an unusually receptive segment of the general stream of thought and seem to strike fire. The elucidation of the causes and possible prevention, control, and cure of rampant killing infectious diseases, with which almost every person was intimately familiar, was one of the last, and caught popular imagination as it developed.

The transition from the specific microbic etiology of fermentations and related phenomena to that of infectious disease was more readily reached than might appear. The analogies between the fermentable mixture and the susceptible individual, between active fermentation and clinical disease, and finally subsidence of active fermentation to recovery from and immunity to infectious disease were obvious. Further, the spread of contagion was already appreciated and something of the nature of the infectious agent indicated by epidemiological evidence as in Snow's studies on cholera, and those of Semmelweiss and Holmes on puerperal sepsis in hospitals.

The implications of Pasteur's studies on fermentation for infectious disease were almost immediately appreciated, notably by Lister, the British surgeon. He applied the basic principles to his own work, controlling infection in the operating room by liberal use of phenol, and so initiated, in 1867, an era of antiseptic surgery with a remarkable reduction in intercurrent infection and mortality. These practices were displaced within the next two decades by those of aseptic surgery, largely by von Bergmann in Berlin, as a consequence of a growing appreciation of the importance of infected persons as the primary source of sepsis.

It remained for the German physician Koch (1843–1910) to develop the experimental methods necessary to proof of a causal relation between bacteria and infectious disease. From the first applications of microscopy to the study of microorganisms it was clear that different morphological types existed and that these occurred in nature in mixed populations for the most part. It was evident also from the early studies on pyemia that morphological criteria did not suffice to differentiate and characterize the bacteria since morphologically identical forms differed markedly in patho-

genic properties. It was essential to separate such microorganisms from one another.

Initially this had been accomplished approximately, as in Pasteur's earliest work, by dilution in liquid culture mediums, and tubes showing growth in high dilutions of inoculum in which the occurrence of growth was irregular in replicates were assumed to contain only one kind of bacterium, descendants of the single viable parent cell present in the inoculum. Probably one of the greatest single contributions to technical bacteriology was the method of isolation in pure culture developed by Koch. It consisted of dilution of the inoculum and culture on a nutrient medium solidified as a gel by the addition of gelatin, and later agar, that served to separate viable cells from one another with the consequence that their progeny developed as discrete masses of cells of single ancestry, a clone in zoological terminology. Modern microbiology was in large part made possible by, and is based upon, this simple technique.

Although the fungus causing favus was described by Schoenlein in 1839, and the yeast-like organism of thrush by Langenbeck in the same year, the specific microbic etiology of infectious disease was first clearly established in studies on anthrax, an epidemic and highly fatal disease of cattle and other domestic animals. The microorganism now known as Bacillus anthracis had been observed by Davaine and Rayer in 1850 and by Pollender in 1849 in the blood and organs of animals dying of anthrax, and the disease was transmitted by Brauell in 1867 by the inoculation of normal animals with infected blood. It is generally conceded, however, that modern medical bacteriology began with Koch's studies on this disease in 1877, for in them he developed conclusive evidence of the causal relation of the anthrax bacillus to the disease of a kind subsequently known as satisfying Koch's postulates, i.e., isolation of the microorganism in pure culture, reproduction of the disease in experimental animals by inoculation with the pure culture, and demonstration of the microorganism in the experimental disease. Thus the kind of evidence required to establish the etiology of infectious disease was delineated, and, when shortly thereafter the solid medium technique for the ready isolation of bacteria in pure culture was developed, the last technical block fell into place.

These advances, coupled with the development of staining methods by Koch, Ehrlich, Weigert, and others, provided a tremendous stimulus to the study of infectious diseases that resulted in an immense accumulation of new knowledge within the ensuing two decades. Koch isolated the tubercle bacillus in 1882 and the cholera vibrio in 1883; Klebs described the diphtheria bacillus in 1883, and it was isolated by Löffler in the following year; Frankel discovered the pneumococcus in 1886, and the meningococcus was isolated in 1887 by Weichselbaum. Kitasato cultivated the tetanus bacillus in 1889, and in 1894 he and Yersin discovered the plague bacillus independently. The discovery of diphtheria toxin in 1888 by Roux and Yersin and of tetanus toxin by Kitasato in 1889 made possible some insight into the means by which bacteria produce disease.

Characterization of the pathogenic bacteria in morphological, physiological, and pathological terms, and study of their persistence under adverse conditions and behavior in the infected host were obvious and essential corollaries. Out of this kind of information there grew an understanding of the basic elements of the spread of infection from man to man, and from animal reservoirs of infection to man, either directly as in tuberculosis or indirectly as in bubonic plague, to give a firm foundation to epidemiology. Thus, while Snow deduced the presence of the causative agent of Asiatic cholera in the feces of diseased persons and its transmission to others by way of a common contaminated water supply in the famous Broad Street Pump epidemic in London in 1854. the nature of waterborne enteric disease became much more clear with the isolation and study of enteric pathogens such as the cholera vibrio and the typhoid and dysentery bacilli.

Effective control was an inevitable consequence of the developing understanding of infectious disease and its dissemination. The application of indicated control measures, chlorination of water supplies, pasteurization of milk, etc., coupled with widespread utilization of methods of artificial immunization, has been astonishingly successful. It has resulted in the virtual disappearance of many of the great killing diseases, such as smallpox, typhoid fever, Asiatic cholera, diphtheria, and plague, and a tremendous reduction in others, such as tuberculosis and

7 VIRUSES

scarlet fever, in many parts of the world. All of this stems in large part from implementation of the principle of specific microbic etiology of infectious disease.

VIRUSES

Early in the study of the etiology of infectious diseases, it became apparent that there were etiological agents of disease, apparently living, in that the diseases could be transmitted indefinitely in series, which could neither be seen with the light microscope nor cultivated apart from living host cells, i.e., were not cultivable on the lifeless mediums that supported the growth of bacteria. Iwanowski in 1892, and Beijerinck in 1899, observed the first of these agents, that causing mosaic disease of the tobacco plant, which Beijerinck described as a contagium vivum fluidum since it was present in bacteria-free filtrates of infected juice. A similar agent causing foot-and-mouth disease of cattle was described in 1897 by Löffler and Frosch, and the causative agent of vellow fever by the American Army Commission under the direction of Reed in 1900. Similar agents producing a transmissible lysis of bacteria were found by Twort in 1916 and d'Herelle in 1917, to give three groups of agents now known as the plant, animal, and bacterial viruses respectively.

The term virus steadily contracted from its meaning of any living agent, including bacteria, that was current prior to 1930, through the limitation filterable (or filtrable) virus because some of these agents would pass bacteria-proof filters, to the present significance of the term after about 1940. The extremely small size of the viruses was inferred from both inability to see them and from the ability of at least some to pass

through fine filters.

Over the next four or five decades accumulated information, based first on the ability of viruses to pass through some but not all collodion membranes of graded porosity, sedimentation rates in high speed centrifuges, and finally direct observation with electron microscope, showed that these agents ranged from a diameter of 200 mu. or just at the limit of resolution of the light microscope, to infectious particles as small as 10 m μ . Further, chemical analyses showed that the larger forms contained pro-

tein, polysaccharide, and lipid in biologically reasonable proportions and thus resembled bacteria, while the smaller forms appeared to be pure nucleoprotein. Some of the latter were, in fact, prepared in crystalline form: the first was tobacco mosaic virus which was prepared as needle-shaped paracrystals in 1935. Subsequently other plant viruses were prepared in the form of true crystals, viz., tomato bushy stunt virus as uniform rhombic dodecahedral crystals, and in 1955 a strain of the poliomyelitis, one of the smallest animals viruses, was prepared as bipyramidal tetragonal prisms. From data such as these, it was obvious that the viruses do not constitute a homogeneous group of infectious agents.

Related microorganisms, perhaps to be regarded as intermediary between the bacteria and the viruses, were described as the causative agents of a number of febrile diseases. The etiological agent of spotted fever was found by Ricketts in 1909, and subsequently da Rocha Lima, von Prowazek. and others showed that similar microorganisms caused fevers of the typhus group, tsutsugamushi and related diseases, and still later Q fever was found to be of similar etiology. These microorganisms, known as rickettsiae, were found to be as large as

small bacteria and so demonstrable under

the light microscope but, like the viruses.

to proliferate only in the presence of appriopriate host cells.

As a consequence of this dependence of the viruses and rickettsiae on living host cells, a variety of methods for their propagation in tissue culture were developed since the early part of the twentieth century. In such tissue cultures the host cells were found to be affected adversely, the cytopathogenic property of viruses, and it has been possible to demonstrate in this way the presence of viruses in materials from apparently normal human beings, especially from the upper respiratory tract and the intestinal tract. Propagation of viruses in the embryonated hen's egg was described in the early 1930's proliferation on the chorioallantoic membrane, in the allantoic cavity or yolk sac, or in the tissues of the embryo itself depending upon the agent and the route of inoculation. With the advent of antibiotics in the 1940's, it became possible to control the contamination of cultures of animal cells and tissues, resulting in the development of readily applicable methods of tissue culture for the propagation of viruses. The stimulus to virology was similar to that given to bacteriology by Koch's method of isolating bacteria in pure culture.

This dependence upon the host cell characterizing the viruses and rickettsiae raised the concept of parasitism to a high degree of intimacy. With one or two minor exceptions, these agents have been found to have no independent metabolism and thus to parasitize the metabolic mechanisms of the host cell, and some of them, particularly the bacterial viruses, appear to consist of little more than genic material which, on entry into the host cell, dominates its normal directive mechanisms to force the synthesis of new virus substance. Put more precisely, the virus parasitizes the high energyyielding reactions and the synthetic functions of the ribosomes of the host cell. It then became possible to show that nucleic acids, separated from the virus particle by phenol extraction or other methods, gave rise to the synthesis of complete virus on introduction into the host cell. Such preparations are known as infectious nucleic acid. The fundamental significance of such observations is obvious, and it has become more and more difficult, if indeed it is possible, to differentiate between the living and the nonliving.

IMMUNITY

It has long been a matter of common knowledge that the individual recovered from infectious disease was often specifically refractory to subsequent attacks of that disease, and that this refractory state, or acquired immunity, persisted in some instances for many years. The practice of deliberate exposure to the infectious agent in order to produce the disease, and therefore immunity, persists to this day in the case of a few diseases such as mumps and measles which are milder and less complicated in children than in adults. It reached a culmination of a sort in the practice of variolation, the deliberate inoculation of a susceptible person with pustular material from an individual with smallpox (variola). The disease so produced was less often fatal than naturally acquired smallpox and provided a more or less permanent protection against it. Variolation had been practiced in the Near East for an unknown time prior to its introduction to Western Europe in 1718 by Lady Mary Wortley Montague, wife of the British ambassador in Constantinople.

Artificial inoculation with an infectious agent of reduced virulence for man to produce a mild infection and consequent protection against naturally occurring disease began with the work of the Englishman Jenner. Noting the infrequent occurrence of smallpox in milkmaids who had been infected with cowpox (vaccinia), he undertook deliberate inoculation of man with vaccinia, followed by inoculation with variola some weeks later. He was able to report in 1796 that such prior infection conferred a high degree of protection against smallpox. This method of vaccination, or Jennerian prophylaxis, was widely practiced thereafter and remains in use today.

With the discovery of pathogenic microorganisms, it became possible to study immunity to infectious disease in a systematic way. Pasteur, working first with chicken cholera and later with anthrax and swine ervsipelas, showed that the basic principle uncovered by Jenner could be generalized to include disease other than smallpox. His most striking application of it was the development of rabies prophylactic, rabies virus that had been reduced in virulence for man by successive passages in the rabbit. Not long afterward the American workers, Salmon and Theobald Smith, found that inoculation with killed microorganisms could also stimulate the development of immunity. In 1890 von Behring and Kitasato laid the basis for antitoxic immunity through their discovery of tetanus and diphtheria antitoxins. This rounded out the approach to effective prophylaxis of infectious disease by acquired immunity produced in response to the artificial inoculation of modified microorganisms, killed microorganisms, or relevant products of microorganisms.

It became evident that the immune animal is characterized by, first, the presence of antibody, reacting specifically with microorganisms and their products, in the body fluids and localized in the globulin fraction

of the serum protein, and second, by an accelerated response of the phagocytic cells to the presence of microorganisms. The former, humoral immunity, was studied intensively by many workers such as Bordet, Ehrlich, and others, and two general approaches may be distinguished. The one has been concerned with the relation of humoral antibody to effective immunity. In some cases, such as antitoxic immunity, this seemed clear, but in others such as antibacterial and antiviral immunity, the immune state is more complex and presents many problems that are still unsolved.

The other has been concerned with the nature of antigenic specificity, which was elucidated largely through the work of Landsteiner in the 1920's, the nature of antibody, and that of the antigen-antibody reaction in physiochemical terms. Serology, the *in vitro* study of antigens and antibodies, led to the development of diagnostic serological reactions such as the Widal and Wassermann tests, and made possible the characterization of microorganisms on the basis of their constituent antigens by application of the methods of antigenic analysis.

The study of immunity associated with the activity of phagocytic cells, or cellular immunity, was developed initially largely by Metchnikoff in the late 1880's and thereafter. Proceeding from studies with *Daphnia*, he expanded the phenomenon of engulfment and destruction of invading microorganisms by polymorphonuclear leucocytes (microphages) and macrophages into a system of immunology. On this foundation Aschoff described and defined the free and fixed tissue phagocytes as the reticulo-endothelial system of cells. Further elucidation of the developmental potencies of cells of lymphoid origin by Maximov led to a general conception of the inflammatory reaction and cellular response to infection as a process of mobilization of phagocytic cells and destruction of microorganisms, followed by tissue repair through the development of macrophages into fibroblasts.

While cellular immunity and humoral immunity developed somewhat apart, it was early apparent that they are both facets of the host defense mechanism, obviously linked by the occurrence of circulating opsonic antibody, and by the intimate relation

of cells of the lymphoid-macrophage system to antibody formation.

CHEMOTHERAPY

One of Paul Ehrlich's many contributions to microbiology around the turn of the century was that to the chemotherapy of infectious disease. He urged the idea of the "magic bullet," a chemical compound, nontoxic to the host, that would search out and kill the invading microorganisms. Substances having a selective antimicrobial activity were known, but had arisen from folklore, or later on a trial-and-error basis not sanctified by age. Ouinine used for the treatment of malaria and representing the active principle of cinchona bark is a notable example of the former, and exploitation of the small antimalarial activity of methylene blue leading to the synthesis of Atabrine is an instance of the latter. Ehrlich implemented this concept by the synthesis and testing of hundreds of compounds of arsenic and eventually arrived at the parent compound salvarsan, which was an effective chemotherapeutic drug for the treatment of African sleeping sickness and syphilis. The antimicrobial activity of antimony was similarly followed out by others to give compounds that are reasonably effective in the treatment of infections with some of the animal parasites, but for many years all efforts to prepare antimicrobial substances effective in the treatment of bacterial infections fruitless.

In retrospect it is clear that the fallacy was the unquestioned acceptance of the working hypothesis requiring that a substance, to have promise, must have bactericidal activity. It became apparent later, and only since the early 1930's, that a bacteriostatic effect inhibiting reproduction of the invading microorganism is sufficient to tip the balance in favor of the host, with actual destruction of the bacteria accomplished by the host defense mechanisms.

Probably the first rational approach to the biological aspects of the problem of the chemotherapy of disease of bacterial etiology was that of Avery in the late 1920's. He put two kinds of information together. The one was the ability of microorganisms in general to decompose any kind of organic substrate, and the other the intimate association between the presence of capsular polysaccharide substance in the pneumococcus and the ability of that bacterium to cause disease. It seemed possible to isolate a microorganism that would specifically decompose the pneumococcal polysaccharide and separate the enzyme, catalyzing that decomposition for use as a therapeutic agent. This was accomplished but, while the cellfree enzyme preparation had therapeutic activity, it was too highly toxic to the host to have practical value.

A variation on a similar theme grew out of the study of microbial antagonisms, the adverse effect of one kind of microorganism on another when grown together in mixed culture. This effect was found to be due, in some instances, to the formation of a specifically toxic substance by the one kind of microorganism. The first of these found, pyocyanin, had in fact been known since the middle of the nineteenth century, prior to the work of Pasteur, Koch, and others, and had been extracted with organic solvents from the "blue pus" characterizing infections with the microorganism forming it.

The great majority of such substances, including pyocyanin, were found to be highly toxic to higher animals, but some proved to be of sufficiently greater toxicity to the microorganism than to the host that effective concentrations could be produced and maintained in the tissues of the infected host. Of these substances, the antibiotics, the first to be discovered was penicillin which was found in 1929, but its possible chemotherapeutic activity was not appreciated until 1940; prior to that time its greatest utility was that of facilitating the primary isolation of microorganisms, such as the influenza bacillus, that are relatively resistant to it. Of the other more widely known antibiotics, streptomycin was found in 1943. chloramphenicol in 1947, and the tetracyclines in 1948, 1950, and 1954. Although resting on a firm biological background, the discovery of these substances still remains on trial-and-error basis in that they are found through methods of screening vast numbers of microorganisms for activity.

The discovery of the chemotherapeutic activity of sulfonamides in the 1930's was also accidental. More than 1000 derivatives

of the parent compound, sulfanilamide, have been prepared, of which perhaps half a dozen are in common use. Since these substances antedated the discovery of the antibiotic chemotherapeutic agents, they were the first effective antibacterial drugs, and the effect of the introduction of these substances was nowhere more dramatic than in the treatment of puerperal sepsis. The fundamental importance of the sulfonamides, however, did not become apparent until 1940.

During the preceding decade a body of detailed information relative to the nutritional requirements of bacteria had been assembled, and study of the nature of the antibacterial activity of the sulfonamides had shown that it was primarily bacteriostatic rather than bactericidal. These apparently unrelated kinds of information were joined in 1940 by Woods's observation that the antibacterial activity of sulfonamides was specifically antagonized by p-aminobenzoic acid, an essential growth factor for some bacteria and utilized by very many if not all in the synthesis of folic acid. The structural similarity of p-aminobenzoic acid to the active portion of the drug molecule, p-aminobenzene sulfonic acid, suggested that the antibacterial activity of the latter was a result of a specific interference with the synthesis of an essential metabolite. This proved to be true and provided the first rational theory of chemotherapy of infectious disease in establishing the general principle of using structural and functional analogues of essential metabolites to exploit small differences, qualitative or quantitative, between the essential metabolic reactions of the host and parasite cells. Its general significance is illustrated in the chemotherapy of neoplastic disease with nucleic acid analogues.

MICROBIAL GENETICS

Variability of microorganisms was obvious from the beginning of their systematic study as alterations in certain of their properties, produced inadvertently as a consequence of maintenance on artificial culture mediums or as changes deliberately induced by laboratory manipulation. Alteration in the properties used for the differentiation

and characterization of microorganisms was found to occur with some facility, not as a consequence of an unusual plasticity, but rather because of their short generation time and occurrence as populations made up of huge numbers of individuals. Because of the relative simplicity of structure of microorganisms, especially the bacteria and viruses, the bacteriologist lacked a comparative anatomy that makes up the foundation of botany and zoology. He was forced to rely on such seemingly ephemeral characteristics as fermentation, nutritive requirements, and antigenic character, all of which varied, and had no means of knowing whether any of these, with the possible exception of the last, were biologically trivial or fundamental.

Studies in the 1920's with yeasts showed that, in these forms at least, the ability to ferment various carbohydrate substrates was genically determined in that a Mendelian segregation of characters of this kind occurred in the exchange of nuclear material during spore formation. This work was largely neglected by bacteriologists, and the demonstration that conjugation occurred between physiologically unlike bacterial cells did not occur until the 1940's.

While the importance of establishing the occurrence of genic control of the kind of characters used for the differentiation and identification of bacteria is unquestionable, microbiology contributed uniquely to, and markedly broadened, the basis of heredity through the occurrence of phenomena hitherto not demonstrable in other forms.

In 1928 it was shown that immunological types of living pneumococci could be changed by growing them in the presence of killed pneumococci of another type. Further refinement made it clear that the active principle in the dead pneumococci was a highly polymerized deoxyribonucleic acid that could be separated from the cells and purified in the test tube before addition to the living culture. This phenomenon was later found to occur also with other kinds of bacteria and is possibly related to the mechanisms of replication of certain of the bacterial viruses whose "invasion" of the host cell was found to consist of "inoculation" of that cell with viral deoxyribonucleic acid, in that in both kinds of phenomenon foreign nuclear material becomes functional in directing the metabolism of the host cell so

that it synthesizes a new substance and persists as an integral part of the genetic apparatus of the recipient.

Still another kind of modification of hereditary mechanisms was described in the early 1950's in which bacterial virus was found to transmit from one bacterial cell to another physiological characters that appear as stable inheritable elements in the new host cell. Finally, it has long been known that alteration in the physiological behavior of bacteria may be induced specifically by the environment even in the absence of cell multiplication, and this phenomenon of enzyme adaptation is conditioned by the specific substrate of each enzyme.

Out of this seeming disorder of microbial variation there has come, by way of analysis on a molecular basis, an orderly array of widely applicable general principles. This has led to a new era of biology, molecular biology, and modern genetics is, in large part, microbial genetics.⁴

RECONSIDERATION

The microbiologist has been something of a renegade, by necessity a master of many trades but a servant of none, and showing a certain lack of respect for the classical and established. Pasteur was not impressed with the opinions of Liebig and Berzelius, nor was it of any concern to Koch that no self-respecting medical man would work with a disease of domestic animals. The microbiologist has taken what he needed from zoology, botany, chemistry, physiology, pathology, medicine, etc., to create a new discipline, and it is apparent from even a cursory survey of the more important developments tracing its growth over a century that remarkable results were achieved.

It is literally true that the majority of the important infectious diseases were conquered in that they have been, or can be, so effectively controlled as to remove all but tuberculosis from the 10 leading causes of death in this and certain other countries, and thus has been made probably the largest single contribution to increased human life expectancy. New industries have been created, notably the fermentation industry with its ramifications into the production of antibiotics and vitamins of microbial origin, and

the biologicals and chemotherapeutic drugs of the pharmaceutical industry. Less obvious, but more important, has been a large group of unique contributions to biology, ranging from the creation of the new science of immunology, through much of biochemical physiology, to a greatly broadened concept of hereditary mechanisms to provide the basis for modern genetics and molecular biology.

Aside from such highly successful practical and theoretical applications, microbiology owes its important position in biological science to its general significance. There is no question but that microbiology has produced a change in man's conceptions of the world around him so sweeping as almost to deserve the term revolutionary. Up to the middle of the nineteenth century the character of many of the most familiar natural processes was entirely misunderstood; contemporary spontaneous generation of at least the lower forms of life was the generally accepted belief; infectious diseases were not differentiated from one another, and the most fantastic hypotheses were advanced to explain their existence.

Although the great mass of material phenomena elsewhere had been brought into apparent orderliness and system, here was a region in which the unscientific imagination rioted in mystery and extravagance. The penetration of this realm of obscurity by the discoveries of microbiologists gave the human race for the first time in its history a rational theory of disease, dispelled the myths of spontaneous generation, and set the process of decay and kindred phenomena in their true relation to the great cycle of living and nonliving matter.

The new conception of the microscopic underworld which microbiology brought into biological science must be reckoned as a conspicuous landmark and, insofar as it has changed the attitude of man toward the universe, may be regarded as one of the most important triumphs of natural science.

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Chapter Two

LABORATORY METHODS*

The study of microorganisms in the laboratory by the various procedures of isolation in pure culture, cultivation, microscopic examination, and characterization by biochemical and immunological methods necessarily begins before detailed consideration of their nature and properties can be completed. The basic laboratory procedures are summarized here in didactic form, and their rationale will become apparent later.

STERILIZATION

The determination of the character and properties of a given species of microorganism is necessarily based on its study in pure culture, *i.e.*, its separation from other microorganisms. Since bacteria and other microorganisms such as fungi are ubiquitous, all material coming in direct contact with the microorganisms under study must be subject to preliminary sterilization. The sterilizing agent most commonly used is heat, dry or moist, but occasionally other methods, such as filtration of liquids through filters which retain microorganisms cultivable in the usual laboratory mediums, are desirable.

Glassware and instruments. The usual glassware includes flasks, petri dishes, test tubes and pipettes which must, of course, be scrupulously clean. Flasks and tubes are plugged with nonabsorbent cotton which prevents entry of bacteria after sterilization, and a cotton plug is inserted in the mouth end of the pipettes.

Petri dishes and pipettes may be placed in cans with covers or wrapped in paper to maintain sterility. Surgical instruments are wrapped in paper or towels, syringes separated and wrapped in paper, hypodermic needles placed in plugged test tubes, and other equipment similarly prepared.

Sterilization is effected in a hot air oven, electric or gas fired. The material to be sterilized is placed in the oven without crowding and the temperature raised to 170° to 180° C. and maintained for a period of not less than two hours. If appropriate temperature control equipment is not available, a slight browning of the cotton plugs is taken as indicative of sterilization, but this is a doubtful procedure.

In some instances sterilization by heating to dull red in a Bunsen burner flame is exceedingly useful as in the flaming of forceps tips, the platinum or nichrome wire needles and loops used for transferring bacteria, and the lips of test tubes before and after transfer of bacterial culture.

Surgical instruments, hypodermic needles and syringes, and similar equipment may be sterilized with respect to the vegetative forms of bacteria by boiling in water or 1 per cent bicarbonate solution for three to five minutes, but this does not suffice to destroy the spores of bacteria and fungi.

Culture mediums and other liquids. Material containing water cannot, of course, be sterilized by dry heat, but moist heat is a more effective sterilizing agent in any case.

Intermittent sterilization. As indicated before, boiling water does not provide a high enough temperature to bring about complete sterilization. Certain kinds of liquid culture mediums, however, are affected unfavorably by moist heat at higher temperatures, and these may be sterilized by intermittent sterilization. This is based on the assumption that the vegetative cells of microorganisms are destroyed at 100° C. in the presence of water, and that the surviving spores will germinate, and these vegetative cells will be destroyed in turn by exposure to 100° C. In practice the material to be so sterilized is exposed to free-flowing steam in an Arnold sterilizer for 30 minutes, removed and incubated until the next day and the steaming repeated, and a third incubation and steaming is included as a safety factor so to speak. The method is not too successful in that spores not infrequently show a delayed germination, and spores of obligate anaerobic bacteria

^{*}For the most part the methods described here are identical with or closely follow those of generally accepted procedures to be found in various compilations included among the references at the end of this chapter.

may not germinate. Sterilization by the intermittent method is, however, seldom required.

Autoclave sterilization. The most efficient method of sterilization is that effected by steam at temperatures above 100° C. which may be produced when the steam is under pressure. The devices employed in the use of steam under pressure include the common pressure cooker, autoclaves of various designs, and dressing sterilizers, which are autoclaves to which vacuum can be applied in order to dry fabrics that have been sterilized. Steam is generated directly in the apparatus or supplied by connection to a high pressure steam line.

As in the case of dry heat sterilization, the material to be sterilized must not be packed too tightly in the autoclave. The temperature is raised to 120° C. and maintained for a period depending on the material to be sterilized. As short a time as 15 minutes suffices for small volumes of material, as in culture tubes, loosely spaced, but if the tubes are packed in baskets, 20 minutes or possibly more should be allowed. For larger volumes, as in flasks, longer periods of exposure are necessary, e.g., for 500 ml. quantities 30 to 40 minutes. The time is that elapsing after the temperature is reached and until the pressure is shut off; the preliminary heating as pressure is built up and the slow fall in pressure at the end are not included. No attempt should ever be made to reduce the pressure rapidly after sterilization, for liquids will boil out of their containers.

Sterilization by filtration. It is often desirable to sterilize solutions such as cultures in liquid mediums, solutions of substances relatively unstable to heat such as certain sugars and the like, without subjecting them to heat at sterilizing temperatures. These may be sterilized by filtration through filters of such fine porosity that most microorganisms are held back. The method of sterilization by filtration is particularly useful in obtaining soluble products such as toxins.

Several kinds of filters are available. The Berkefeld filters are made of infusorial earth in three porosities, V (viel) or coarse, N (normal), and W (wenig); of these the V filters remove most but not all bacteria, the N filters usually sterilize, and the W filters are used for very small microorganisms. The Chamberland filter, of unglazed porcelain, is made in graded porosities, L1, L2, L3, etc., and of these the L3 is roughly equivalent to the Berkefeld N. The Seitz or Seitz-Werke filter is made of metal, and the filtering element is an asbestos pad which is the equivalent of the Berkefeld N filter. Sintered glass filters are available in a variety of porosities which in the Pyrex filters are designated C (coarse), M (medium), F (fine), and UF (ultrafine). The UF filter is bacteria-proof while the others are not. Cellulose ester membrane filters, marketed as Millipore filters, are available in a variety of pore sizes; type HA, with a pore diameter of 0.45 μ , is sterilizing filter.

Filtration is accomplished by a pressure differential which may be obtained by positive pressure on the liquid to be filtered, or negative pressure on the filtrate, and usually a differential of 20 to 30 cm. Hg suffices for reasonably rapid filtration without foaming. The Berkefeld and Chamberland types are prone to develop leaks, either as cracks in the filter candle, or where the candle is cemented to the metal portions, and the integrity of the filter must be controlled frequently by including a trace organism such as Serratia marcescens in the liquid to be filtered and by culture of the filtrate.

Whatever filter is used it must, of course, have been sterilized, usually in the autoclave, prior to use.

PREPARATION OF CULTURE MEDIUMS*

The nutritive requirements of microorganisms vary greatly; some will grow readily in the so-called synthetic mediums containing inorganic salts, including an ammonium salt, together with a simple organic compound such as glucose or asparagin as a source of carbon and energy. In general, microbiological mediums have been developed on a trial-and-error basis about a basic nutrient medium containing peptone and the water-soluble material, largely extractives, of muscle tissue. The source of the latter may be commercial preparations of meat extract, but a somewhat better medium is obtained if these substances are extracted from fresh meat, beef or veal for the most part.

The basal nutrient solution may be modified in a variety of ways. Thus, it may be solidified by the addition of gelatin or agar. or enriched with serum, ascitic fluid, defibrinated blood, and the like, to support the growth of the more fastidious bacteria. Various sugars may be added, together with an acid-base indicator, for the determination of the fermentative properties of bacteria; nitrate or tryptophan may be added to test for ability to reduce nitrate to nitrite, form indol from tryptophan, etc. Or culture mediums may be prepared which are differential and accentuate physiological differences, and specific inhibitory agents may be added to produce selective mediums which allow the growth of some kinds of microorganisms and suppress that of others. These various characteristics may be combined, of course, as in the case of an enriched medium which is both selective and inhibitory. Consequently, there is a great variety of bacteriological culture mediums, but they are in large part interrelated modifications of a basal nutrient solution. The constituents and method of preparation of a few of the

^{*}Many culture mediums are available commercially in dehydrated form, requiring only solution in water, dispensing, and sterilizing. Certain commonly used agar mediums are similarly available as poured plates or slants in plastic disposable "glassware."

commonly used culture mediums are reviewed here.

Basal mediums

Meat extract broth and agar. The medium usually has the following composition:

meat extract	3 gm.
peptone	5 gm.
(NaCl	5 gm.)
(agar	
distilled water	1000 ml.

It is necessary to include the sodium chloride if the medium is to be used for serological work or enriched with blood, and the agar is omitted if a liquid medium is desired. The ingredients are dissolved in water. Agar has the property of not dissolving or of its gels not melting unless it is heated to boiling, but its solution does not gel until the temperature reaches about 40° C. If agar is used it is necessary to boil the solution or heat it in the autoclave and clarify by filtration. The initial solution is usually slightly acid, and the reaction is adjusted to pH 7 by the addition of alkali.

Meat infusion broth and agar. The infusion mediums differ from the extract mediums in that the extractives are obtained by infusion of fresh meat, but otherwise the composition is the same, viz...

extract of 400 to 600 gm. fresh lean beef	or veal
peptone	
(NaCl	5 gm.)
(agar	
distilled water to	1000 ml.

The meat is ground in a meat chopper, suspended in 1 liter of distilled water, and infused overnight in the refrigerator. The following morning the fat is skimmed off with absorbent cotton and the infusion squeezed through muslin and made up to 1000 ml. The other ingredients are added, the reaction adjusted to pH 7, the solution heated to 100° C. for about 20 minutes, regardless of whether or not agar is used, filtered through coarse paper, and made up to 1000 ml. The heating is for the purpose of coagulating the tissue proteins dissolved during infusion so that they can be removed by filtration.

Mediums for biochemical tests

Sugar broths. The sugar broths are meat extract or meat infusion broth to which the required carbohydrate has been added to a concentration of 1 per cent. Unless the carbohydrate is stable to autoclave sterilization, it must be sterilized separately by filtration in concentrated solution and added aseptically to the sterile broth. It is customary to add an acid-base indicator dye, bromthymol blue or bromcresol purple, and the medium may be dispensed in culture tubes containing an inverted vial that fills during sterilization and serves to collect gas evolved in the fermentation of the sugar.

Nitrate broth. To test for ability to reduce nitrate, bacteria are cultured in nitrate broth, infusion or extract

broth containing 0.1 per cent KNO₃. The culture is tested for the presence of nitrite with sulfanilic acid and α -naphthylamine reagents.

The sulfanilic acid reagent is prepared by dissolving 8 gm. of sulfanilic acid in 1000 ml. of 5N acetic acid. The α -naphthylamine reagent (α -amidonaphthalene acetate) is prepared by dissolving 5 gm. of α -naphthylamine in 1000 ml. of 5N acetic acid and clearing by filtration through absorbent cotton.

To test for nitrite add 2 ml. of each of the two reagents to 3 to 5 ml. of culture; the development of a rose color, the nitroso reaction, indicates the presence of nitrite.

Tryptophan broth. The test for the production of indol from tryptophan can often be carried out with cultures in meat broth or peptone water, the latter containing peptone but no meat extract or infusion. The important factor is an adequate amount of tryptophan in the peptone. Some peptones are deficient, but a peptone marketed under the name Tryptone contains adequate amounts of the amino acid.

The presence of indol is tested for after two to four days' incubation of the culture by layering a small amount of Ehrlich's reagent on the surface of the culture; the development of a red color at the interface indicates the presence of indol. If a color does not appear within one minute, add a small amount, equal to that of the Ehrlich reagent, of saturated aqueous solution of potassium persulfate.

Ehrlich's reagent consists of 4 gm. of p-dimethylaminobenzaldehyde dissolved in a mixture of 380 ml. of ethyl alcohol and 80 ml. of concentrated hydrochloric acid.

Lead acetate agar. This medium is meat extract or meat infusion agar containing 0.05 per cent basic lead acetate and dispensed in culture tubes without slanting. It is usually prepared by mixing equal amounts of sterile double strength basal medium, i.e., containing double amounts of the ingredients, and sterile 0.1 per cent aqueous solution of basic lead acetate.

The medium is inoculated by stab, and the production of hydrogen sulfide from the sulfur-containing amino acids of the peptone is indicated as a blackening or browning of the medium.

Meat extract and meat infusion gelatin. These mediums are the basal mediums solidified by the addition of 12 per cent gelatin and are dispensed in culture tubes without slanting and inoculated by stab. They indicate whether or not gelatin is liquefied by the microorganism under study. Gelatin liquefies at 37° C., and gelatin cultures should be incubated at 22° C. In the case of those bacteria which will not grow at this lower temperature, the culture may be incubated in the usual way and after incubation tested for ability to solidify by chilling. Gelatin liquefaction sometimes occurs slowly, and cultures should be retained for not less than two weeks unless positive earlier.

Milk. Milk is an adequate culture medium for many bacteria without modification other than the addition of an indicator, bromcresol purple or litmus in an amount of 5 ml. of a 0.25 per cent alcoholic solution per liter. Fresh skim milk may be used, but skim milk powder is usually more convenient. One liter of medium is made from 150 gm. of milk powder, first rubbed up in a mortar and then diluted to volume. Medium prepared from powdered milk may be autoclaved, but that from fresh milk is best sterilized by the intermittent method

Because of protein and carbohydrate content milk cultures undergo a variety of biochemical changes, viz.,

- (1) The development of an alkaline reaction, usually after three to four days' incubation.
- (2) The development of an alkaline reaction with precipitation of the casein as a rennet curd, with or without reduction of the indicator.
- (3) The development of alkalinity and rennet curd precipitation, followed by peptonization or digestion of the curd, resulting in a clearing and brownish discoloration; the indicator is usually reduced by the time digestion is apparent.
- (4) Acid formation, usually in 24 hours, with or without reduction of the indicator.
- (5) Acid formation and precipitation of the casein, usually with reduction of the indicator.

Enriched mediums. Basal infusion mediums may be made richer by the use of special peptones, such as neopeptone and proteose peptone, and the addition of small amounts of glucose and phosphate buffer. In addition, fluids such as serum, ascitic fluid, or defibrinated blood may be added.

Blood agar. The most common and most useful enriched medium is blood agar-veal or beef infusion base to which defibrinated whole blood is added. The blood is usually taken from the rabbit, sheep or horse under sterile conditions and defibrinated as it is drawn by shaking with glass beads. The sterile infusion agar base is liquefied by heating, cooled to about 45° C., the sterile blood added to 10 per cent concentration, and the medium dispensed before it solidifies, usually in petri dishes or as slants in culture tubes. The entire operation is carried out under sterile conditions and the medium incubated for 24 hours to detect contamination, and then stored in the refrigerator. This medium is rich enough to support the growth of almost all the fastidious pathogenic bacteria and has the advantage that hemolysis is shown directly in the cultures.

Cystine blood agar. This is simply blood agar medium further enriched by the addition of 0.01 per cent cystine and 1 per cent glucose to the basal medium prior to the addition of blood as above. It is useful primarily in the cultivation of Pasteurella tularensis.

Chocolate agar. This medium is heated blood agar and is particularly useful for the cultivation of the gonococcus and meningococcus. Formulas vary somewhat, some workers preferring a beef heart infusion (see below) instead of muscle tissue infusion and proteose peptone instead of peptone. The basal infusion medium is enriched by the addition of defibrinated blood as in the preparation of blood agar, but after the addition of the blood it is heated slowly in a water bath until the medium has a chocolate brown color; if the heating is excessive the blood coagulates and the finished medium will contain clumps of cooked blood, so the temperature should not rise above 75° C, at the most.

Other infusions. The use of infusions of tissues other than beef or veal muscle is sometimes desirable for the cultivation of certain bacteria. Beef liver infusion is prepared in the same manner as that described for the other infusions and, for culture of Brucella for which liver infusion medium is particularly useful, the basal medium is enriched by the addition of 1 per cent egg albumin. Beef heart infusion is also prepared in the same manner as beef muscle infusion, and the basal medium completed by the addition of a peptone, sodium chloride, etc.

Löffler's medium. This medium is used primarily for culture of the diphtheria bacillus and consists of three parts of sterile beef serum and one part of meat infusion basal medium containing I per cent glucose. The serum is added to the sterile base, mixed, dispensed in culture tubes, slanted, and sterilized by the intermittent method. Sterilization may be carried out on three successive days in the autoclave at 15 pounds steam pressure without letting the air out, or it may be held at 15 pounds for 15 minutes and the air allowed to escape slowly while pressure is maintained and, when all the air has escaped, holding for an additional 15 minutes, and after sterilization allowing the pressure to fall very slowly. The medium is solid because of coagulation of the serum, and sterilization must be carried out carefully to avoid the formation of bubbles.

Blood culture medium (Kracke). This is a highly enriched medium containing beef heart infusion and brain suspension and is used primarily for blood culture of pathogenic bacteria in infections accompanied by bacteremia. Its composition is:

heart infusion	750 ml.
brain suspension	250 ml.
sodium citrate	i gm.
dextrose	10 gm.
peptone	10 gm.
dibasic sodium phosphate	2 gm.
sodium chloride	4 gm.

The heart infusion is prepared in the same manner as muscle tissue infusion described earlier. The brain suspension is prepared by macerating 500 gm. of fresh brain in water, straining through a metal strainer, and heating slowly to boiling with constant stirring. The heating coagulates the brain tissue and leaves it in a state of fine suspension; it must not be filtered. The other ingredients are added to the mixture of heart infusion and brain suspension, the pH is adjusted to 7.4, the mixture is dispensed in 50 ml. amounts in 100 ml. flasks (to leave room for an inoculum of 10 to 20 ml. of patient's blood) and sterilized by autoclaving. Cultures in this medium are essentially enrichment cultures and should be subcultured on appropriate agar mediums such as blood agar for streptococci and staphylococci and on liver infusion agar for Brucella.

Dorset's egg medium. This medium is used for the cultivation of the tubercle bacillus and consists of four fresh eggs and 25 ml. of 0.95 per cent sodium chloride solution. The eggs are scrubbed with a brush in soap and water, rinsed, dried, put into a wire basket and dipped into 95 per cent alcohol, drained, and the remaining alcohol ignited. They are then broken, using aseptic technique, and the whites and yolks placed in a sterile container. Twenty-five ml. of sterile salt solution is added, the whole mixed well with a sterile egg beater, dispensed in culture tubes and slanted, and sterilized by the intermittent method described for Löffler's medium.

Differential and selective mediums. The various differential and selective mediums have been designed to facilitate the isolation of one kind of bacteria in the presence of many other kinds, by accentuating physiologic differences and/or inhibiting the growth of the unwanted forms. Of the pathogenic bacteria, those most commonly encountered admixed with large numbers of closely related forms are the enteric bacilli, and many of the selective mediums are selective for these bac-

teria. The simplest of the purely differential mediums consist of meat extract or infusion agar base together with a sugar and an indicator. The most satisfactory indicator in most cases is bromthymol blue, and the colonies of the sugar-fermenting bacteria are yellow while those of the nonfermenters are blue. Of the selective agents bacteriostatic dyes are often used, and others include bile, tellurite, etc.

Endo's medium. This consists of meat extract agar to which has been added lactose and Schiff's reagent, basic fuchsin decolorized with sulfite. When the lactose is fermented aldehyde intermediates restore the color to the fuchsin and colonies of lactose-fermenting bacteria are red while those of the non-lactose-fermenters are white. The composition of the medium is:

hot melted extract agar	1000 ml.
sodium carbonate, 10% aqueous	10 ml.
lactose	10 gm.
sodium bisulfite, 10% aqueous	10 ml.
basic fuchsin, 3% alcoholic	10 ml.

The pH of the medium is raised by the carbonate and should be 7.6 to 8.0.

Eosin—methylene blue agar. This medium is sterile meat extract agar to which is added 5 ml. of a 10 per cent solution of lactose, 2 ml. of 2 per cent aqueous eosin, and 2 ml. of 0.5 per cent aqueous methylene blue. These solutions are added aseptically to the melted and cooled medium, and the mixture is dispensed in sterile petri dishes.

Deoxycholate-citrate medium. This medium is differential in that it contains lactose and neutral red as an indicator, and selective in that it contains bile salt, and is one of the most satisfactory and widely used mediums for the isolation of Salmonella and dysentery bacilli. It contains:

meat infusion	1000	ml.
peptone	10	gm.
agar	20	gm.
lactose	10	gm.
sodium citrate	20.6	gm.
sodium deoxycholate	5	gm.
lead chloride, 0.35% aqueous	1	ml.
ferric ammonium citrate	2	gm.
neutral red, 1% aqueous	. 2	ml.

The complete medium is not stable on storage. The peptone and meat infusion are mixed, the pH adjusted to 7.5. The solution is boiled for three minutes, lost water added, and filtered through paper. The agar is added to the hot solution together with 5 ml. of N NaOH, and it is allowed to stand for 15 minutes, then steamed at 100° C. for 20 minutes. Then the lactose, citrate, deoxycholate, and lead chloride are added. This may be stored and remelted for use. Just before use, the agar is heated to 100° C., the ferric ammonium citrate added, and the pH adjusted to 7.4 using phenol red as an indicator, and finally the neutral red is added and the medium dispensed in plates. Note that it is not autoclaved.

Bismuth sulfite medium (Hajna). This medium is used primarily for the isolation of the typhoid bacillus from fecal specimens and is highly specific for that organism. As originally developed it was difficult to prepare consistently, but Hajna's modification is highly satisfactory. It consists of a meat extract agar base con-

taining 0.5 per cent glucose, the bismuth sulfite mixture, and brilliant green. The bismuth sulfite mixture is prepared as follows:

- Dissolve 80 gm. of anhydrous bismuth sulfite in 400 ml. of hot water with stirring.
- (2) Make a paste of 24 gm. of bismuth citrate in 40 ml. of water, add 12 ml. of concentrated ammonia, and stir until a sol is formed. Dilute to 200 ml. with distilled water and mix to give a solution.
- (3) Mix (1) and (2), and add 42 gm. of anhydrous dibasic sodium phosphate and mix until dissolved.
- (4) Dissolve 4 gm. of ferrous sulfate in 50 ml. of distilled water containing 2 drops of concentrated hydrochloric acid, and add 40 ml. of this solution to mixture (3). Mix and boil gently for about 2 minutes until slate gray in color. This is the bismuth sulfite mixture, and it is stable for about 2 months.

For use, add 70 ml. of the bismuth sulfite mixture and 4 ml. of a 1 per cent aqueous solution of brilliant green to each 1000 ml. of the hot melted agar base and autoclave for not more than 10 minutes; then dispense in petri dishes. The complete medium may be kept for perhaps two weeks in the refrigerator without autoclaving and should be autoclaved just prior to dispensing.

Tetrathionate enrichment broth. Nutritive enrichment broths are used for preliminary culture of specimens followed by subculture on selective differential mediums. Tetrathionate broth and selenite F broth (see below) are very useful in the isolation of Salmonella from fecal specimens. The active agents in tetrathionate broth are bile salts, brilliant green, and tetrathionate, the last formed by oxidation of thiosulfate with iodine just prior to inoculation. Its composition is as follows:

Ţ	proteose peptone	5	gm.
ł	bile salts (Bacto)	1	gm.
. (distilled water	1000	ml.
. (calcium carbonate	10	gm.
	sodium thiosulfate	30	gm.
ł	brilliant green, 1% aqueous	11	ml.
i	iodine, 25% aqueous 2.5 ml. pe	r 100	ml.
	[iodine	25	gm.
	potassium iodide	20	gm.
	water		ml.

The peptone and bile salts are dissolved in water and calcium carbonate added, and autoclaved. The sodium thiosulfate and brilliant green are added to the sterilized medium, and it is dispensed in tubes or small flasks. Just prior to inoculation add the iodine solution in the proportion of 2.5 ml. per 100 ml.

Selenite F broth. This medium contains selenite as an inhibitory agent and its composition is:

sodium hydrogen selenite	4 gm.
peptone	5 gm.
lactose	4 gm.
sodium phosphates (anhydrous)	10 gm.
distilled water	1000 ml.

It is necessary to determine the proportions of acid and basic phosphates which will give a final reaction of pH 7.0 to 7.1 with the peptone and the lot or brand of selenite used. The ingredients are dissolved in warm water and then brought to a boil and dispensed. The medium is not autoclaved.

Chocolate-tellurite medium. This is a chocolate agar prepared by adding 10 per cent of defibrinated blood to meat infusion base agar as described earlier but modified by the addition of 150 ml. of sterile 0.3 per cent aqueous solution of potassium tellurite to every 1000 ml. of infusion base. It is used for culture of diphtheria bacilli.

Petragnani's medium. This is a medium used for culture of tubercle bacilli and contains malachite green to inhibit the growth of gram-positive bacteria and molds. Its composition is:

milk	900 ml.
potato flour	36 gm.
peptone	6 gm.
pieces of potato, size of an egg	6
eggs	24
egg yolk	6
glycerin	72 ml.
malachite green, 2% aqueous	60 ml.

The potatoes are cut into thin slices, the milk, potato flour, and peptone added, and the whole cooked in a double boiler for two hours with stirring. The eggs and egg yolks are broken together, the glycerin and malachite green added, and the mixture shaken well. The milk-potato mixture is cooled to 50° C., the egglycerin-dye mixture added and mixed well, and the whole is filtered through gauze, dispensed into tubes, slanted, and sterilized by the intermittent method described for Löffler's medium.

MICROSCOPIC EXAMINATION

The direct observation of microorganisms is an essential part of their study. Characteristics such as the shape and grouping of the cells, the presence or absence of structures such as capsules, flagella, and spores, the reaction to differential stains, and the like, are of considerable differential significance.

MOTILITY

Some bacteria have flagella which function as organs of locomotion and are analogous to the cilia of protozoa. The absence, or presence and number and location of flagella, is a characteristic of differential value. Flagella are too small to be seen in the usual stained preparations, but may be demonstrated by special staining procedures in which dye is deposited on them. The demonstration of flagella by staining methods gives results which are too uncertain to be useful as a routine procedure. Their presence, though not number and position, may be

inferred from a demonstration of motility, *i.e.*, a movement of translocation as differentiated from the random motion of brownian movement. This may be carried out in two ways, *viz.*,

- (1) Motility may be observed directly in suspensions of young cultures (usually not more than 18 hours old) suspended in a drop of saline or broth on a coverglass inverted on a hollow ground or "well" slide (the hanging drop preparation). Unstained bacteria are difficult to see, and the amount of light should be reduced to a minimum to give maximum resolution. Observation is facilitated by focusing the microscope on the edge of the drop and then moving the slide so that the drop is within the field of observation.
- (2) Motility may also be demonstrated by stab culture in "motility medium," a soft (0.5 per cent) agar nutrient medium dispensed in culture tubes. The medium is inoculated with the straight wire in a single stab penetrating several centimeters into the medium. Following incubation, the growth of nonmotile bacteria is found to be confined sharply to the path of the inoculating needle, while that of motile bacteria tends to diffuse out from the line of inoculation to give a fuzzy streak of growth.

STAINING

Bacteria may be stained or dyed with aniline dyes, more readily with the basic dyes.⁵ Staining may be with a single dye, or simple stain, most commonly crystal violet, methylene blue, or basic fuchsin, with mixed dyes or polychrome stains, or by differential methods based on the relative affinity of different bacteria or different structures of the bacterial cell for the stains used.

The so-called Romanovsky or polychrome stains are relatively complex mixtures of dyes that stain the constituents of protoplasm in contrasting colors-generally nuclear material red and cytoplasm blue. They are used primarily for staining blood and blood parasites such as trypanosomes and malarial parasites. In general, they consist of oxidation products of methylene blue (methylthionin) together with the parent substance and the compounds formed between these basic dyes and the acid dye eosin (eosinates). Of these, Giemsa stain is one of the most widely used. American Giemsa contains Azure A (asymmetrical dimethylthionin) eosinate, Azure B (trimethylthionin) eosinate, methylene blue eosinate and methylene blue chloride dissolved as a stock solution in methyl alcohol and glycerol. The material to be stained is commonly fixed in

methyl alcohol, and stained by flooding with a freshly prepared 1:10 dilution of stock solution of Giemsa.

The nature of the staining process has been a matter of considerable controversy, in large part as to whether it is fundamentally a physical or chemical process. It is now generally agreed that it is chemical in nature and the mechanism an ionic interchange between the basic dye and the acidic portions of the protoplasm—nucleic acids and their compounds—with the formation of insoluble dye-nucleotide compounds that do not diffuse out of the cell.

Stain solutions. The composition of stain solutions varies considerably in the literature. The several, frequently somewhat indefinite, formulas have been interpreted by the Biological Stain Commission and are given here in the emended form recommended by that Commission. In older formulas the dye content is often given as milliliters of a saturated alcoholic solution, and the accompanying table gives the solubilities of those most commonly used, in water and in 95 per cent alcohol.

Löffler's alkaline methylene blue

Solution A methylene blue (90% dye con-		
tent)ethyl alcohol (95%)	0.3 30	gm. ml.
Solution B		
dilute KOH (0.01% by weight)	100	ml.
The two solutions are mixed in the abov	e quant	ities.

Ziehl's carbolfuchsin Solution A

basic fuchsin (90% dye content)	0.3	gm.
ethyl alcohol (95%)	10	ml.
Solution B		
phenol	5	gm.
distilled water	95	ml.
Mix the two solutions in the above quant	ities.	

The Gram stain

Δ	١N	4 N	40N	IUM	OXALATE	CRYSTAL	VIOLET (HUCKER!

Solution A crystal violet (90% dye con-		
tent)	2	gm.
ethyl alcohol (95%)	20	ml.
Solution B		
ammonium oxalate	0.8	gm.
distilled water	80	ml.
Mix.the two solutions in the above quant	ities.	
0		

SAFRANIN

safranin (2.5% s	olution in 95%	* * * * * * * * * * * * * * * * * * * *	
		10	ml.
distilled water		100	ml

Albert's diphtheria stain (Laybourn)

toluidine blue	0.15	gm.
malachite green	0.02	gm.
acetic acid (glacial)	1	ml.
ethyl alcohol (95%)	2	ml.
distilled water	100	ml.

Preparation of smears. Slides should be clean and free from all grease, and may be cleaned in soap and water, thoroughly rinsed, and stored in alcohol or xylol. Before use a slide is passed through the Bunsen burner flame two or three times. A drop of distilled water is placed on the cooled slide and a small amount of bacterial growth suspended in it, spread, and allowed to dry in air. The film is then fixed by passing through the burner flame two or three times, or, for some stains, by immersion in absolute methyl alcohol, glacial acetic acid, or other fixatives.

Procedure of the simple stain. The fixed smear is covered with stain, allowed to stand for 30 to 60 seconds, rinsed off with tap water, excess water removed by blotting, and allowed to dry in air. Bacteria are usually examined under the oil immersion objective, and the oil can be placed directly on top of the smear.

The Gram stain. Of the differential stains, Gram stain is one of the most valuable and most generally applied. The procedure is

Solubilities of Common Bacteriological Stains

COLOR		PER CENT S	PER CENT SOLUBLE IN		
INDEX NUMBER	NAME	WATER	95% ALCOHOL		
655	Auramine O	0.74	4.49		
681	Crystal violet (chloride)	1.68	13.87		
676	Fuchsin, basic (pararosanilin hydrochloride)	0.26	5.93		
657	Malachite green (oxalate)	7.60	7.52		
922	Methylene blue (hydrochloride)	3.55	1.48		
841	Safranin	5.45	3.41		
925	Toluidine blue O	3.82	0.57		

essentially one of staining with crystal violet, mordanting with iodine solution, decolorizing with alcohol, and counterstaining with a dye of contrasting color. The stain was originally developed by the histologist Gram in an effort to stain bacteria in tissues differentially and it has been subject to many modifications. By this procedure bacteria are separated into two groups, those which retain the crystal violet and are said to be gram-positive, and those which are decolorized and stain with the counterstain and are gram-negative. The distinction is not always sharp for there is considerable variation in ease of decolorization, some gram-positive bacteria such as the pneumococcus become gram-negative after they die, and some bacteria are gramvariable. This last group is not large enough to detract from the practical value of the stain.

Both the crystal violet and the iodine are highly specific while the alcohol and the counterstain are not. Almost all other dyes, even methyl green which differs by only one methyl group from crystal violet, are unsuitable, either being retained or removed by alcohol whether or not the iodine is applied. A few other reagents, notably HgCl₂, may be substituted for iodine, and stannous chloride, pyrogallol, and freshly prepared solutions of hydroquinone show some slight activity.

However arbitrary Gram's method of staining may appear, the reaction is apparently associated with fundamental differences between the gram-positive and gram-negative organisms. There is, for example, a pronounced correlation between it and resistance to the bacteriostatic action of certain dyes and antibiotic substances and to the action of other chemical and physical agents. The nature of the Gram reaction has, therefore, been of considerable interest (see Chap. Three).

Procedure of the Gram stain. In addition to crystal violet and safranin staining solutions, Gram's* iodine solution is required as a mordant. Its composition is:

iodine	 . 1 gn	n.
potassium iodide	 . 2 gn	n,
distilled water	 . 300 ml	I.

^{*}This reagent is frequently given as Lugol's iodine; Lugol's iodine is defined in the U.S.P., and in the Gram stain is used as a 1:15 dilution.

The staining procedure is as follows:

- (1) The heat-fixed smear is stained for one minute with ammonium oxalate crystal violet.
- (2) Wash in tap water.
- (3) Flood with Gram's iodine solution and allow to stand one minute.
- (4) Wash in tap water and blot dry.
- (5) Decolorize 30 seconds with gentle agitation in 95 per cent alcohol, and blot dry.
- (6) Counterstain 10 to 30 seconds in safranin.
- (7) Wash in tap water, blot dry, and examine.

Acid-fast stain (Ziehl-Neelsen method). Certain bacteria, characterized by a high lipid content, cannot be stained by the usual procedure of the simple stain, and either heat or prolonged contact is required to drive the stain into the cells. Conversely, the stained forms are equally difficult to decolorize and resist decolorization with acid alcohol. These organisms are designated acid-fast and include the tubercle bacilli and related forms, the leprosy bacillus and certain of the actinomycetes.

Staining procedure

- Stain the smear for three to five minutes in steaming Ziehl's carbolfuchsin. An alternative procedure useful in diagnostic laboratories is to stain in the cold for 18 to 24 hours.
- (2) Rinse in tap water.
- (3) Decolorize in 95 per cent ethyl alcohol, containing 3 per cent by volume of concentrated hydrochloric acid, until only a suggestion of pink color remains.
- (4) Wash in tap water.
- (5) Counterstain for 30 to 60 seconds with alkaline methylene blue.
- (6) Wash in tap water, dry, and examine.

Fontana stain for spirochetes. The spirochetes stain very poorly or not at all by the usual simple staining procedure and are usually stained by a silver impregnation method.

Preparation of ammoniacal silver nitrate solution. Dissolve 5 gm. silver nitrate in 100 ml. of distilled water. Remove a few milliliters, and to the rest of the solution add, drop by drop, concentrated ammonia solution until the sepia precipitate which forms is dissolved. Then add, drop by drop, enough more of the silver nitrate solution to produce a slight cloud which persists after shaking. This solution is stable for some months.

Staining procedure

- Steam the heat-fixed smear in a solution of 5 per cent tannic acid in 1 per cent phenol for 30 seconds.
- (2) Wash for 30 seconds in running water.
- (3) Flood with ammoniacal silver nitrate solution, heat gently, and allow to stand 20 to 30 seconds after steaming has begun,
- (4) Wash in tap water, blot dry, and examine. The spirochetes appear as dark brown or black on a dark maroon field.

Capsule stain (Hiss's method). The bacterial capsule does not stain in the simple stain or Gram stain pro-

cedures. A number of methods of staining the capsules have been used of which that of Hiss is one of the most simple and effective. The stain is an aqueous solution of basic fuchsin or crystal violet, 0.15 to 0.3 per cent and 0.05 to 0.1 per cent respectively.

Staining procedure. The bacteria should be grown in ascitic fluid or serum medium for maximum capsule development, and bacteria grown on solid mediums should be suspended in serum for preparation of the smear. The smear is air-dried and fixed by heat.

 Stain with either aqueous basic fuchsin or aqueous crystal violet by heating gently until the stain steams.

- (2) Wash off the stain with 20 per cent aqueous copper sulfate solution
- (3) Blot dry and examine.

The bacterial cells are deeply stained and the capsules a faint blue or pink, depending upon which stain has been used.

Spore stain. Like acid-fast bacteria, spores are very difficult to stain and appear as unstained in the usual stained smear. The stain may be driven in by heat, and the Ziehl-Neelsen acid-fast staining procedure may be used with the modification that decolorization is less rigorous, *i.e.*, 95 per cent alcohol or absolute acetone should be used instead of acid alcohol.

Culture of Bacteria

Methods of obtaining pure cultures. When fluid culture mediums are inoculated with substances such as soil, water, or excreta, many kinds of organisms develop simultaneously side by side, and a heterogeneous mixture, or mixed culture, of bacteria results. Any technical procedure for obtaining pure cultures is dependent upon the isolation of a single viable bacterium which is allowed to multiply in a suitable culture medium. The first reliable method of isolation of pure cultures was devised by Koch in 1881. This method has proved so satisfactory that it has been employed to the present day with only minor modifications. If nutrient gelatin or agar is inoculated while fluid (for example, at 42° C.) and is then solidified and kept under favorable temperature conditions, many of the living bacteria that have been introduced are able to multiply. Since the bacteria cannot move about freely, but are fixed in the stiffened medium, the progeny of each organism form distinct masses or colonies. These colonies consist of many millions of bacteria and are readily visible to the naked eye or by means of a low power hand lens. If the colonies are not closely crowded, a pure culture may be obtained by touching a colony with the tip of a sterile needle and inoculating tubes of fresh culture mediums. In order to secure a large surface upon which the colonies shall be spread out and made easily accessible, the gelatin or agar after inoculation is poured while still fluid into sterilized flat shallow dishes (petri dishes) fitted with glass covers.

It will be clear that a given colony may arise from two or more parent cells if these remain attached or close together in the agar medium, and the colony will not be a pure culture in the event that the juxtaposed cells are of different species. This possibility has been investigated by McNew⁶ using the plant pathogen *Phytomonas stewarti*. He found that 98 per cent or more of the colonies arose from single cells, hence the probability of a mixed culture in a single colony is 0.02 or less. Successive plating or picking a number of colonies makes a pure culture a practical certainty. It is likely, however, that this figure differs for different bacteria, *i.e.*, staphylococci are more likely to remain attached than micrococci or bacilli.

It is self-evident that the pure culture so obtained is not only homogeneous with respect to kind or species of bacteria, but since the microorganisms are descendants of a single parent cell, they constitute what the zoologist or protozoologist terms a clone.

Technique of making plate cultures. Three tubes of agar (1, 2, 3), melted at 100° C., are placed in a water bath at 42° C., a temperature that is just above the solidifying point of agar and is not injurious to bacteria. Tube I is inoculated with a loopful of the material to be plated. The cotton plug is then replaced and the contents of the tube are mixed by carefully tilting back and forth and rotating the tube on its long axis. From this tube two loopfuls of agar are transferred to tube 2, and after mixing, two more loopfuls carried from tube 2 to tube 3. The contents of the several tubes are then poured into petri dishes. As soon as the cotton plug is removed, the mouth of each tube should be passed through the flame and inserted under the edge of the lifted petri dish cover and the agar quickly poured out. The covered petri dish may then be tipped cautiously back and forth to distribute the agar evenly before it solidifies. Agar plates placed in the incubator after solidification should be inverted in order to avoid spreading of the growth

through condensation of moisture on the surface of the medium. Even if there are a great many bacteria in the original material, the plate from tube 3 will probably contain the organisms in small enough numbers to develop well-isolated colonies. On the other hand, if there are very few bacteria in the material inoculated, plate 1 will probably present more satisfactory conditions. Gelatin plates are made in the same manner as agar except that gelatin may be cooled as low as 25° C. without solidifying.

Under exceptional conditions, such as work in the field, when petri dishes are not available, so-called roll tubes may be made. The tubes containing the liquid agar medium are tilted until the agar almost reaches the cotton plug. The tubes are then rotated in this position against a block of ice, and when the process is complete the test tube is coated on the inside with a thin layer of solid medium. In this way a considerable surface is obtained, and, after incubation, colonies are readily picked.

Quantitative dilution. It is sometimes of advantage before plating to make accurate dilutions of highly polluted fluids, such as sewage, in order to get colonies few enough in number to be well isolated. If there is reason to suppose that the number of bacteria is 200 per ml. or more, 1 ml. of the sample is mixed with 9 ml. of sterile water. If a higher dilution is required, proceed in a similar manner. See below.

One milliliter of each dilution is placed in a sterile petri dish, and 6 to 8 ml. of liquid cooled agar medium is added. The two are thoroughly mixed by tilting the petri dish back and forth and then allowed to solidify. After incubation the total number of colonies on the plates containing 50 to 200 colonies is counted, and the total number is multiplied by the dilution to give the number of organisms present in the original sample. Such bacterial counts are relatively inaccurate, errors of 10 to 15 per cent being common in even the most careful work, and must be regarded only as useful approximations.

Streak plates. Not infrequently mediums used for the isolation of the more fastidious bacteria are such that it is technically difficult to make the usual pour plates. The most common of these mediums are those which contain fresh blood. Such mediums are ordinarily prepared in quantity and sterile plates poured. These may be stored in the icebox until used. Dilution and consequent isolation of bacteria may be accomplished with these mediums by a process known as streaking. A sterile wire loop is dipped into the bacterial suspension and rubbed back and forth on the surface of the solid medium, making successive streaks as close together as possible. After incubation it will be found that at some point sufficient numbers of bacteria were rubbed off the loop to allow the development of individual colonies in the path of the streak. These may be picked and transferred to fresh mediums. Quantitative dilutions may be prepared in sterile broth or saline and 0.5 ml. of each dilution pipetted onto the surface of the medium and spread evenly by appropriate tilting.

Carbon dioxide tension. A number of bacteria, particularly the gonococcus, meningococcus, and Brucella, require an increased tension of carbon dioxide over that of ordinary atmosphere, commonly 10 to 15 per cent. Cultures of such bacteria are incubated in airtight jars in which a part of the air has been replaced by carbon dioxide. One or another of three methods is commonly used.

- (1) The most obvious and effective method is direct replacement in which the jar is a vacuum desiccator that is partially evacuated, 9 to 10 cm. Hg negative pressure, and the evacuated air replaced with carbon dioxide from a tank of the compressed gas. The method has the disadvantage that it requires a certain amount of equipment.
- (2) A simple and equally effective method is that of putting a short candle stub in the jar with the cultures, lighting it, and closing the jar. The candle burns for a short time, forming carbon dioxide, and then goes out. The partial pressure of carbon dioxide obtained is not subject to control, but the precise tension is not too important, and adequate amounts are formed.
- (3) Carbon dioxide may also be generated from sodium bicarbonate by the addition of sulfuric acid. The amounts required must be calculated on the basis of the size of the jar used so that 10 to 15 per cent of the total gas volume is the evolved carbon dioxide.

Cultivation of obligate anaerobes. Atmospheric oxygen is toxic to a group of bacteria designated the obligate anaerobes, which includes the bacilli of tetanus and gaseous gangrene, the botulinus bacillus, the Bacteroides, and anaerobic cocci. Various methods have been devised for the cultivation of these bacteria which center about the elimination of gaseous oxygen and which may be grouped under four general heads.

(1) Enriched mediums in deep tubes. Perhaps the simplest method is that of the so-called shake culture which is in an agar medium liquefied and boiled to drive out dissolved oxygen, inoculated when cool but still liquid, and solidified quickly in cold water. This is the basis of culture in the Veillon tube, a glass tube stoppered at both ends, which is sometimes used

(A)	To dilute	1:	10	use	1	ml.	of	sample	to	9	ml.	of	serile	water.
(B)	** ;	1:	100		ě.					99				
(C)	**	1: 1,	,000		çç		44	(A)	46	99	44			
(D)		1: 10	,000		46		44	(B)	• •	99				
(E)	"	1: 100.	,000		44		"	(C)		99	- 64		. 44	44
(F)	"	1:1,000	,000		6,6		"	(D)		99	44			

in the isolation of obligate anaerobes in pure culture; the tube may be broken or the column of agar medium forced out into a sterile petri dish and cut at the various points where isolated colonies occur deep in the medium.

A somewhat simpler method of culture in liquid medium is based on the use of a rich medium to which is added a piece of fresh sterile tissue such as rabbit kidney. Such mediums show strong oxygen uptakes as a result of the respiration of the fresh tissue, and the depths of the medium are anaerobic. As soon as growth is initiated the evolution of gas, usually from contained glucose, is vigorous enough to prevent solution of atmospheric oxygen, and the culture remains anaerobic.

(2) REMOVAL OF OXYGEN BY COMBUSTION. Oxygen may be removed from the gaseous environment of cultures incubated in sealed jars in a number of ways. Sufficiently anaerobic conditions can frequently be obtained by saturating a piece of filter paper with alcohol, placing it in the jar and igniting, and sealing the jar immediately. Similarly, a small metal container filled with chalk may be put into a jar, a piece of phosphorus placed in it, and the jar sealed immediately. The phosphorus pentoxide formed will dissolve in a small amount of water placed in the bottom of the jar and is not harmful to bacterial cultures, but the phosphorus begins to burn again when the jar is opened, and the method is somewhat dangerous.

By far the most convenient combustion method is that in which the oxygen is removed by combination with hydrogen in the presence of heated platinized asbestos. Jars designed for this purpose with platinized asbestos heated by a small electric coil have been described by Brewer and are termed Brewer anaerobic jars. The jar rim is sealed against both positive and negative pressure and hydrogen or illuminating gas run in to give some positive pressure. The electric current is turned on, and the oxygen is burned out in the presence of the combustible gas and catalyst. This method is considerably more reliable than the combustion methods described above but is somewhat dangerous; if the platinized asbestos is finely divided, heating is unnecessary.

(3) CHEMICAL ABSORPTION OF OXYGEN. Contained oxygen in sealed jars may also be removed by chemical methods, of which the most widely used is a mixture of pyrogallic acid and alkali in the bottom of a vacuum desiccator. The jar should be closed immediately on addition of the reagents, and it is preferable to mix after closing by tilting or spilling one reagent into the other. Excess alkali is to be avoided. The reagents are 20 per cent aqueous sodium hydroxide and 40 per cent aqueous pyrogallic acid mixed in proportions of 5 ml. of alkali to 2 ml. of pyrogallic acid. The alkali-pyrogallic acid method is applicable to single culture tubes; a stopper of absorbent cotton is pushed down to leave about an inch of space above it, pyrogallic acid and sodium hydroxide placed in this space to saturate the cotton, the tube closed at once with a tight-fitting rubber stopper and turned upside down and incubated in that position. It may be used also for the anaerobic incubation of a single petri dish in combination with the Spray dish, which is a false bottom, so to speak, over which the medium-containing half of the petri dish is inverted and sealed. The Spray dish is divided by a ridge in the center; 4 ml. pyrogallic acid is put on one side and 10 ml. of alkali on the other; after sealing on the petri dish bottom, the two are mixed by tilting.

(4) DISPLACEMENT OF AIR BY AN INERT GAS. The air contained in an anaerobic jar may be mechanically displaced by an inert gas, commonly hydrogen since tanked nitrogen contains sufficient oxygen to inhibit the growth of the fastidious obligate anaerobes. A jar with two connections, one reaching to the bottom, designated a Novy jar, may be used with tanked hydrogen. The hydrogen is run in from the bottom of the jar until the contained air is displaced. The air in an anaerobic jar may also be displaced by hydrogen evolved in the reaction between sulfuric acid and powdered chromium metal.

Control of anaerobiosis. It is usually desirable to include an indicator of anaerobiosis in an anaerobic jar to indicate that anaerobic conditions have been obtained and are maintained, i.e., there is no leakage of atmospheric oxygen into the jar during incubation. The usual indicator is a tube of methylene blue prepared as follows: equal parts of N/160 sodium hydroxide, 0.015 per cent methylene blue and 6 per cent sterile aqueous solution of glucose are mixed, boiled just before the jar is to be sealed until the methylene blue is reduced, and the open tube put into the jar. So long as it remains colorless anaerobic conditions are maintained.

SYSTEMATIC STUDY OF BACTERIA IN PURE CULTURE

The characteristics of bacteria that are of significance in their identification must be studied in some uniform fashion, not only with regard to the methods used but also as to the characteristics that shall be considered of first importance. Although the classification of bacteria is in a somewhat dubious state, this uncertainty has resulted largely from a relatively hazy comprehension of the fundamental biologic significance of some differential characteristics and consequent doubt as to the proper relative weight to be assigned to them in the arrangement of the finer subdivisions of taxonomic schemes. The main lines of approach, however, are clear, and whatever their relative biologic significance may prove to be, the readily determinable characteristics of bacteria serve the practical purpose of distinguishing these microorganisms.

There are four general categories which, in the order of the fineness of distinction they make possible, are: (a) morphology, both gross and microscopic; (b) physiologic capabilities in terms of biochemical reactions; (c) pathogenicity for experimental animals; (d) immunological character.

The preliminary and basic study of a bacterium lies in the systematic determination of its cultural characters. The most important of these are:

(A) Morphology

- gross morphology—that of colonies of the organism with respect to size, texture, color, shape, etc.
 - (a) on nutrient or infusion semisolid mediums(b) on special mediums

(2) microscopic morphology, including

(a) size, shape, and grouping of the organisms

(b) presence or absence of spores

(c) motility

(d) presence or absence of capsule

(e) staining reactions

(B) Biochemical reactions

- (1) the fermentation of sugars, usually dextrose, lactose, and sucrose, although others may be included together with the hydrolysis of starch
- (2) liquefaction of gelatin

(3) formation of indol

(4) reduction of nitrate to nitrite

(5) production of hydrogen sulfide

(6) special biochemical tests, such as the Voges-Proskauer reaction, the methyl red test, hemolysis on blood agar, etc.

Such preliminary examination ordinarily affords a great deal of information about a given bacterial culture. The feasibility of additional study rests firmly on the foundation laid down by the studies outlined above.

Various attempts have been made to systematize the study of bacteria. One of the most successful of these has resulted from the efforts of a Committee on Bacteriological Technique of the Society of American Bacteriologists (now the American Society for Microbiology).

ANIMAL INOCULATION

An indispensable adjunct to the study of the pathogenic bacteria is the experimental animal. Animals are used not only for the study of the pathology of infectious disease and as an aid in the isolation of some bacteria in pure culture, but also for the experimental production of immune serums and studies on the various manifestations of immune phenomena. The maintenance by animal passage of such infectious agents as the viruses, which cannot be cultivated on lifeless mediums, is common also. The animals generally used are rabbits, guinea pigs, white mice, and white rats, although in special cases others, such as rhesus monkeys, are necessary.

Routes of inoculation. Experimental animals may be inoculated by a variety of routes, usually one or another being prefer-

able under the particular circumstances. The common routes of inoculation are intradermal, subcutaneous, intramuscular, intraperitoneal, and intravenous. Other routes such as intraocular, intracerebral, and intrathecal may be desirable at times. In any case, inoculation is carried out with a syringe and hypodermic needle, the capacity of the syringe and size of the needle used depending upon the quantity of material to be injected and the route of inoculation. The site of inoculation must be prepared by removal of the hair by shaving or a depilatory, followed by disinfection by swabbing with alcohol, tincture of iodine, etc. The syringe and needles have, of course, been previously sterilized by dry heat or by boiling.

Intracutaneous inoculation. Material inoculated intracutaneously is inoculated into the skin. A fold of skin is pinched up and the needle inserted, lumen up, as superficially as possible. A raised white spot showing the pits of the hair follicles indicates a successful injection. Not more than 0.2 ml. may be inoculated by this route.

Subcutaneous inoculation. Subcutaneous inoculation requires considerably less skill on the part of the operator. A fold of skin is pinched up as before and the needle inserted through the skin to its full length. The injected material forms a bleb or blister. The amount that may be injected by this route depends upon the size of the animal.

Intramuscular inoculation. In the intramuscular inoculation, the needle is inserted deep into the muscular tissue, usually the posterior muscles of the thigh or the lateral thoracic or abdominal muscles.

Intraperitoneal inoculation. Intraperitoneal inoculation is carried out as one would a subcutaneous injection on the abdomen except that after the needle penetrates the skin, it is held at right angles to the peritoneal wall and thrust through into the peritoneal cavity.

Intravenous inoculation. Intravenous inoculation may be carried out in a variety of ways, the choice depending upon the size of the animal. Rabbits are conveniently inoculated in the marginal vein of the ear. The needle is inserted through the skin and into the vein from the side. If the needle is within the vein, blood will disappear in the vein from the point of the needle onward as the material is injected. Needless to say, the injection should take place in the direction of blood flow. Guinea pigs may be inoculated into the large superficial vein on the dorsal and inner side of the hind leg or into one of the external jugular veins. Both require anesthesia of the animal and a small incision, which may be later closed with a stitch or collodion. Rats may be inoculated in an external jugular vein and mice in one of the lateral veins of the tail.

Drawing blood. Blood may be taken from the rabbit from the marginal vein of the ear or directly from the heart. The appropriate area is shaved and swabbed with alcohol. If blood is to be taken from the ear, the marginal vein is cut across with a razor blade and the blood allowed to drop into a centrifuge tube or test tube. Flow may be hastened by preliminary application of a towel wrung out of hot water to induce hyperemia. When

blood is to be taken directly from the heart, a 30 to 50 ml. syringe is used and the needle inserted between the ribs at the point of maximum pulsation and thrust into the heart, care being taken to injure the pericardium as little as possible. Blood is most conveniently taken from smaller animals by this means.

Postmortem examination. The autopsy of experimental animals dead of infection is often highly desirable. Not only may cultures be taken, but the gross and microscopic pathology is usually of significance. The animal is fastened down on a board, ventral surface up. It is good practice to have the board in a larger enameled tray to reduce to a minimum the danger of spreading infectious material. When cultures are to be taken, the animal must be opened with sterile instruments. Usually several pairs of scissors and forceps and two or three scalpels are necessary. The site of inoculation is examined for ulcerative or other changes, and the hair and skin are disinfected with cresol or Lysol.

The skin is incised from the pubis to the neck and cut at right angles at the ends of the incision. The two flaps may be laid back by separation of the cutaneous from the underlying muscular tissue with a scalpel. The condition of the subcutaneous tissue and of axillary and inguinal lymph glands may be noted. The peritoneal fluid may be cultured by searing a small area on the surface of the abdomen with a hot spatula and puncturing with a syringe and needle or a Pasteur pipette into which the fluid may be aspirated. The abdominal cavity is opened with fresh instruments, and the flaps are laid back as above. The condition of the abdominal organs, such as spleen and liver, may be noted and cultures taken by first searing the surface and then either puncturing and aspirating fluid with a syringe and needle or a Pasteur pipette, or by removing a small piece of tissue and placing it in nutrient medium. Likewise, pieces of tissue may be removed and placed in fixing fluid for subsequent sectioning. Again with fresh instruments, the costal cartilages are cut to make a V-shaped incision, and this flap is laid back over the head, exposing the thoracic cavity. Any gross pathology may be noted, cultures may be taken from heart's blood and elsewhere if desirable, and pieces of tissue removed for fixation and sectioning. The animal is disposed of by incineration.

It is obviously impossible to define postmortem procedure rigorously. The lymphatic system of guinea pigs dead of tuberculous infection will be of considerable interest. Those dying of diphtheritic toxemia or infection will show characteristic lesions of the adrenals. In some diseases the important postmortem findings may be in the brain and spinal cord. The routine of examination will be determined, therefore, by the particular disease under consideration or, if this is not known, by the symptoms exhibited by the animal before death.

A number of precautions must be observed in the autopsy of animals dead of infectious disease. If cultures are to be taken, fresh sterile instruments must be used at each stage. Instruments once used should be returned to the boiling water sterilizer or placed in tray containing Lysol or, if to be used again, laid down on the edge of the tray with all sharp edges and points inward. Extreme care must be taken to avoid disseminating infectious material. With certain highly contagious diseases such as tularemia, rubber gloves may be desirable. Boiling water sterilization (five minutes) of used instruments is sufficient except with the sporeforming organisms such as anthrax and tetanus, in which case the instruments should be allowed to stand in a solution of Lysol or cresol.

Propagation of Viruses and Rickettsiae*

The viruses and rickettsiae are set apart from the bacteria by their inability to proliferate except in the presence of living host cells (Chap. Four). The propagation of these microorganisms under experimental conditions in the laboratory, apart from infection of the intact susceptible animal, is dependent upon the use of such living host cells. Appropriate host cells are most readily available as the variety of cells and tissues of the embryonated hen's egg, and as mammalian cells of varied origin maintained as cell or tissue cultures.

THE EMBRYONATED EGG^{3, 13}

The sterile, rapidly multiplying, and diversified cells of the embryonated egg include those of the investing membranes as well as those of the embryo proper. The

*This section was prepared by Dr. Dorothy Hamre.

former may be infected by inoculation into the surrounding fluid, as by inoculation of the yolk sac or the allantoic cavity, or directly as in the case of the chorioallantois. The variables, dependent upon the agent being studied, include the age of the embryo, the type of diluent used for the inoculum, the route of inoculation, and the temperature of incubation after inoculation. For example, the typhus rickettsiae are grown in the yolk sac of the six- to eight-day embryo, influenza virus in the allantoic cavity of the 10- to 11-day embryo, and vaccinia and herpes simplex viruses on the chorioallantoic membrane of the 12- to 13-day embryo. Or the embryo may be inoculated directly, as for instance intracerebrally or intravenously, or may be removed, minced and grown as a tissue culture (see below).

Fertile eggs from disease-free flocks should have white shells to facilitate observation by transillumination. When they are to be used for the propagation of rickettsiae or viruses of the psittacosis-lymphogranuloma venereum group, the flock should not be fed diets supplemented with the broad-spectrum antibiotics. The eggs can be stored, prior to incubation, for as much as a week at 5° to 20° C. with little loss of viability. The eggs are incubated at 37° to 37.8° C. at a relative humidity of 40 to 60 per cent in a forced draft incubator, *i.e.*, a conventional egg incubator. On the fifth day, they may be candled and nonviable embryos and infertile eggs discarded. After appropriate further incubation, the egg may be inoculated by any of several routes.

Yolk sac. *Inoculation*. A small hole is drilled or punched, without rupturing the shell membrane, in the center of the shell over the air space at the large end of the egg. After disinfection of the area with iodine-alcohol, the inoculum is introduced by means of a syringe with a 22 g. 1½-inch needle, which is inserted until the tip of the needle is about in the center of the egg. Amounts of 0.25 to 1.0 ml. can be injected. After the needle is withdrawn, the area around the hole is again disinfected and the hole sealed off with melted paraffin, collodion, or nail lacquer.

Harvest. After disinfection by swabbing with iodinealcohol, the shell over the air sac is removed with sterile scissors or forceps. The exposed shell membrane and chorioallantoic membrane are cut away, and the contents of the egg tipped into a sterile petri dish, with a long forceps guiding the material. The yolk sac is then dissected away from the other membranes and placed in a second petri dish which has been weighed. After determination of the wet weight of the yolk sac, it is transferred to a sterile flask or bottle partially filled with glass beads. A suitable volume of diluent is added, and the flask is closed with a sterile rubber stopper and shaken by hand or on a bottle shaker for about 20 minutes. The resulting suspension is centrifuged to remove cellular debris, and the supernatant fluid, containing the virus, is decanted.

Amnionic cavity. Inoculation. The eggs are candled, the air space outlined, and the position of the embryo noted. The shell over the air sac is disinfected and a hole about 1.5 cm. in diameter cut in the center with sterile scissors. The shell membrane is moistened with saline to make it transparent, and a small hole made in the chorioallantois over the embryo with fine-tipped forceps such as iris forceps, taking care not to rupture large blood vessels. The amrionic membrane is picked up with the forceps and brought up through the hole in the chorioallantois in a tentlike fold. The inoculum is injected with a syringe and 25 g. needle into the fold. The membrane is then released, and will drop back into its original position. The hole is sealed with strips of Scotch tape; the gummed surface of this tape is sterile as it is peeled from the roll.

Harvest. The taped shell down to the edge of the air sac is cut off and the exposed shell and chorioallantoic membranes removed with sterile scissors. The allantoic fluid is removed with a pipette. The amnionic membrane is then picked up with sterile forceps and the fluid inside, containing the virus, is removed with a capillary pipette. If there is very little fluid, the sac can be washed out with a suitable diluent. If desired, the embryo may then be removed and appropriate tissues harvested.

Allantoic cavity. Inoculation. The egg is candled, the edge of the air sac outlined, and a line intersecting the edge made at a position where there are no large blood vessels. A small hole is drilled on this intersecting line about 3 mm. above the edge of the air sac. The inoculum, usually 0.2 ml., is introduced by means of a syringe and 25 g. needle, which is inserted through the hole to a depth of about 1 cm. in a line parallel to, but not touching, the shell. The hole is disinfected and sealed with paraffin, collodion, or nail lacquer.

Harvest. The eggs are chilled overnight at 4° C., or for a shorter time in a freezer, to prevent bleeding. After the shell over the air sac is disinfected, it is removed and the exposed shell membrane over the choricallantoic membrane carefully stripped away with sterile

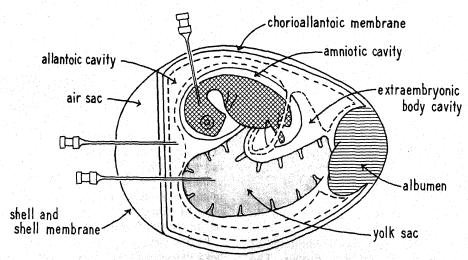


Figure 2. Diagrammatic representation in sagittal section of the embryonated hen's egg 10 to 12 days old. The hypodermic needles show the routes of inoculation of the yolk sac, allantoic cavity, and embryo (head). The chorioallantoic membrane is inoculated after it has been dropped by removing the air from the air sac.

forceps. In the chorioallantoic membrane, away from major blood vessels, a hole is made with a sterile applicator stick, which is then used to hold the embryo and membranes away from the pipette used to remove the allantoic fluid containing the virus.

Chorioallantoic membrane. Inoculation. The egg is placed horizontally on a tray, and a new air sac made by dropping the chorioallantoic membrane as follows: After disinfection of the site, a small hole is made in the center of the large end of the egg over the air space. A second hole is drilled, or punched with a dull punch, collared to prevent penetration of more than 0.8 mm., in the center of the uppermost portion of the egg. It is essential that neither the shell membrane nor the chorioallantoic membrane be damaged in this process. A drop of saline is placed over the hole, and the shell membrane carefully broken by means of a sterile penpoint with a small ball tip or slightly upturned tip; the penpoint is inserted through the drop of saline between the shell and the shell membrane, and then slightly depressed and removed. This usually produces a very small rent in the shell membrane. Then slight suction with a rubber bulb over the hole in the large end of the egg will result in the chorioallantoic membrane's dropping away from the shell. The inoculum is introduced through the hole above the dropped membrane with a syringe and 25 g. needle. The hole is then sealed with Scotch tape.

Harvest. The shell above the dropped membrane is disinfected and removed with sterile scissors to expose the membrane. The chorioallantoic membrane is cut out and placed in a sterile petri dish, and spread out and examined for lesions before weighing and grinding. After grinding in a sterile mortar with a small amount of sterile alundum, the appropriate diluent is added, mixed well with the ground tissue, and the suspension centrifuged to remove debris. The supernatant fluid contains the virus.

TISSUE CULTURE^{9, 14}

Although animal cells and tissues have been grown in culture for many years, it was not until the antibiotics became available to control the vexatious problem of contamination that successful culture could be carried out generally. As now practiced for the propagation of viruses, tissue culture can be carried out in almost any microbiological laboratory provided the necessary attention is given to exceptional cleanliness of glassware and avoidance of bacterial and fungal contamination. The former includes washing glassware in special detergents and rinsing it in double-distilled or distilled and deionized water, while the latter involves use of a hood or small cubicle in the laboratory away from drafts, together with antibiotics in the salt and nutrient solutions.

Culture methods in which the cells are grown on the surface of glass test tubes or bottles are the most frequently employed, and will be described briefly here in terms of examples. Details of these and other tissue culture methods may be found elsewhere. The solutions required are buffered saline and balanced salt solutions, and growth and maintenance mediums.

Salt solutions. One of the most commonly used buffered saline solutions is the phosphate-buffered saline (PBS) of Dulbecco and Vogt. It has the following composition:

(1)	NaCl	8.0	gm.
11.	KCI	0.2	gm.
	Na ₂ HPO ₄	1.15	gm.
	KH ₂ PO ₄	0.2	gm.
	double-distilled water	800	ml.
(2)	CaCl ₂	0.1	gm.
	double-distilled water	100	ml.
(3)	$MgCl_2 \cdot 6 H_2O \dots$	0.1	gm.
	double-distilled water	100	ml.

The three solutions are sterilized separately by autoclaving, and mixed when cool.

The balanced salt solutions (BSS) usually used are Hank's or Earle's; the composition of these is given in the accompanying table. Usually phenol red in a concentration of 1 to 5 mg. per cent is added as an acid-base indicator. The BSS are bicarbonate buffered, and therefore must be sterilized by pressure filtration to avoid loss of carbon dioxide. It follows also that culture tubes and bottles must be tightly stoppered to maintain acid-base equilibrium, and when tissues are grown in petri dishes, an atmosphere containing 5 per cent carbon dioxide is required.

Culture mediums. The culture mediums may resemble bacteriologic mediums as in the case of 0.5 per cent lactalbumin hydrolysate in Hank's or Earle's BSS. Other basal mediums are chemically defined, such as Medium 199 of Morgan, Morton and Parker, and Eagle's basal medium. There

Balanced Salt Solutions (grams per liter)

	EARLE'S	hank's
NaCl KCl CaCl ₂	6.80 0.40 0.20	8.00 0.40 0.14
$MgSO_4 \cdot 7 H_2O$ $Na_2HPO_4 \cdot 12 H_2O$ $NaH_2PO_4 \cdot H_2O$	0.20	0.20 0.12 -
KH₂PO₄ NaHCO₃ Glucose	2.20 1.00	0.06 0.35 1.00

are many such basal mediums for tissue culture.

Growth mediums. To support the growth of tissues, such basal mediums require supplemental serum, usually to 10 per cent, the species of serum depending upon the kind of cells being cultured.

Maintenance mediums. When the tissue culture is fully developed, e.g., a continuous sheet of cells covers the glass surface of the kind of culture described here, the growth medium is removed and a maintenance medium substituted during the period of viral replication. In maintenance mediums, the serum added to the basal medium is reduced in amount, or even omitted in some cases, the precise conditions varying with the kind of culture.*

To all of these solutions and culture mediums antibiotics must be added to prevent microbial contamination. These are: penicillin G, 100 units per ml.; streptomycin, 100 µg. per ml.; and Fungizone, 1 µg. per ml.

Preparation of tissues. Two types of tissue cultures are most often used in virus studies, viz., primary cultures which are cultures of tissue or organ explants that tend to die out on subculture and are necessarily short term, and cultures of continous cell lines which can be carried indefinitely by serial subculture. Here monkey kidney tissue culture will be taken as an example of a primary culture, and that of the HeLa cell as one of the many cell lines.†

Monkey kidney tissue culture. The capsule, connective tissue, and blood vessels in the pelvis are cut away from the kidney and discarded. With sharp scissors or two scalpels the tissue is cut into small pieces about 2 to 5 mm. in size. During this mincing process the tissue is kept moist with PBS, and washed frequently with larger quantities of PBS to remove as much blood as possible.

The minced tissue is transferred to a flask and 50 to 75 ml. of a warm (37° C.) 0.25 per cent solution of trypsin in PBS without calcium, and magnesium (i.e., solution (1) above), is added. The flask is incubated at 37° C. for 15 minutes, the supernatant trypsin poured off and discarded, and fresh trypsin is added, together with a Teflon-covered stirring bar. The flask is placed on a magnetic stirrer at room temperature, and the tissue suspension stirred for 10 minutes at a speed sufficient to keep the fragments of tissue in constant

motion without foaming. The supernatant trypsin, which now contains kidney cells, is decanted, fresh warm trypsin solution is added, and the process repeated.

Meanwhile the supernatant suspension of cells in trypsin is centrifuged at 700 rpm for 10 minutes to pack the cells. The supernatant is removed and discarded, and the cells are suspended in about 10 ml. of growth medium (0.5 per cent lactalbumin hydrolysate in Hank's BSS with 5 per cent calf serum). Each supernatant suspension of trypsin-released cells is treated in this way, and when the minced tissue has been completely digested, the suspensions of resuspended cells are pooled and filtered through three layers of gauze into a 250 ml. graduated centrifuge tube. The cell suspension is centrifuged at 700 rpm for 20 minutes, and the packed cell volume read.

The cells are now suspended in growth medium, using 300 ml. of medium for each ml. of packed cells. This suspension is transferred to the culture vessels. When these are test tubes, 16×125 mm., 0.5 ml. is added to each tube, and the tubes are incubated in a slanted rack so that the cells grow on the side of the tube. If prescription bottles are used, they must contain cell suspension to cover the flat side not more than 5 mm. deep. The uppermost side of the tube or bottle is marked so that, after examination, it can be returned to the incubator in the same position.

Observation of the progress of these cultures can be made with an ordinary compound microscope, using the 10× objective. The tubes or bottles are placed on the microscope stage with the cell layer uppermost. Since this drains the medium from the cells, prolonged observation should be avoided. When the desired cell population is reached, usually after five to seven days incubation at 37° C., the cultures are washed with warm Hank's BSS, and maintenance medium added, 1 ml. to each tube or corresponding amounts to bottle cultures. The maintenance medium, such as Medium 199 without serum, should be one that favors the growth of the virus under study. These cultures are now ready for inoculation with virus, or bottle cultures can be trypsinized as described below for HeLa cell cultures and more cultures prepared; these are often called secondary cultures.

HeLa cell culture. Stock cultures of HeLa cells are usually carried in prescription bottles and transfers made at five to seven day intervals when the cells have formed a confluent sheet on the side of the bottle. Cultures should not be kept longer than seven days because the cell population becomes too large, exhausting the medium and declining in viability.

For subcultures, the growth medium is removed and discarded, the cells washed with two changes of warm Hank's BSS, and sufficient 0.1 per cent trypsin in PBS without calcium or magnesium to cover the cells is added. After incubation at 37° C, for about five minutes, the cells float into suspension on slight agitation of the bottle. The cells are sedimented by centrifugation at 700 rpm for 10 minutes, the supernatant trypsin solution is discarded, and the cells are suspended in Eagle's medium, containing 10 per cent human serum by repeated pipetting. The cell suspension is counted in a hemocytometer, and diluted appropriately for use as an inoculum. Cultures in 16 × 125 mm. test tubes are inoculated with 50,000 cells in 1 ml., and 8 oz. prescription bottles, used for stock cultures, are seeded with 106 cells.

For virus propagation, the freshly seeded cultures

^{*}Many of the solutions and mediums used for tissue culture can be purchased as sterile stock solutions to be diluted in double-distilled water, or complete and ready to use. Sterile serums of many species are also available commercially.

[†]Such cell lines, and some primary tissue cultures such as monkey kidney, can be obtained commercially.

SEROLOGY

are incubated until sufficient cell growth has occurred, usually about four days. The growth medium is discarded and the cells washed three times with warm Hank's BSS, and maintenance medium added. The me-

dium may be Eagle's medium with 5 per cent chicken serum, or another of the mediums devised for this cell line which is best suited for growth of the virus.

29

Immunological Methods

The immunological or serological reactions serve to differentiate bacteria on the basis of their constituent antigens. Such immunological differences are usually, though not always, subordinate to the biochemical differentiation of species, and in general serve to subdivide species into immunological types or varieties. The immunological identification of an unknown microorganism is of considerable diagnostic utility and is frequently of very great value in epidemiological studies in tracing the source and dissemination of infection. Furthermore, serum may be tested against a known antigen to establish the presence of the homologous antibody and thus by inference the presence, immediate or past, of the microorganism or its products in the host, and therefore serves as an indirect diagnostic procedure. The specific neutralization of toxin by antitoxin in vivo serves to measure antibody in serum or the circulating antibody in the living host, and in the latter is a measure of antitoxic immunity. Of the in vitro immunological reactions, those of agglutination of a bacterial or other discrete antigen, precipitation of a soluble antigen, and complement fixation in the presence of soluble or discrete antigen are of general utility.

Titration of diphtheria antitoxin by the Römer method. The intradermal inoculation of diphtheria toxin in the rabbit produces local erythema, and central necrosis with larger doses, which may be used in the assay of toxin, and of toxin in the presence of antitoxin. The end point ordinarily taken is the smallest amount of toxin that, in a dose of 0.2 ml., will produce a zone of erythema 10 mm. in diameter 48 hours after inoculation. This is designated the minimal reacting dose or MRD, and the activity of a toxin may be measured as MRD per ml.

Such a titration measures the potency or toxicity of the toxin preparation, but not its combining power with respect to antitoxin. An expression of the activity of the toxin in the presence of antitoxin is the definition of the Lr dose. This is that amount of toxin which, when mixed with 1 unit of standard antitoxin, contains 1 MRD of unneutralized toxin. It will be clear that a toxin so standardized with respect to standard antitoxin can then be used to titrate an unknown antitoxin by using a constant amount of toxin mixed with serial dilutions

of the unknown antitoxin and testing of the mixtures for activity.

One application of this technique is the titration of the antitoxin content of the serum of persons, immunized, convalescent, or normal. This will be considered here since it illustrates all of the principles involved. In studies on human immunity the usual procedure is to titrate the serum against an amount of toxin which will just give the end point reaction when mixed with 0.002 units of standard antitoxin; this is commonly spoken of as titration at the 1/500th level.

Standardization of toxin at the 1/500th level. Toxins vary widely in potency and combining power, and no precise quantitative procedure may be given. The general model of the titration is as follows:

 Assume that a toxin whose L+ dose is known is available. Then the following preliminary titrations can be carried out:

(a) The standard antitoxin is diluted so that it contains 1 unit per ml.

(h) The toxin is diluted so that it contains 1 L+dose per ml.

(c) The toxin is titrated in dilutions of 1:100, 1:200, 1:300, up to 1:1000 against constant amounts of antitoxin, viz., 1 ml. of diluted antitoxin is mixed with 1 ml. of each toxin dilution, incubated as indicated below, 0.2 ml. of each inoculated intradermally in the rabbit, and the reaction read in 48 hours. This titration determines the Lr dose of toxin.

(d) Additional preliminary titration indicates the approximate amount of toxin required to just give the skin reaction in the presence of 1/500 unit of antitoxin. A more precise determination is carried out as follows:

 Prepare a dilution of the original toxin in sterile broth to contain exactly 100 Lr per ml. This is the so-called stock solution and is relatively stable if prepared from stabilized (aged) toxin.

(ii) Dilute 1 ml. of the stock solution to toxin with 99 ml. of buffer solution to give a solution containing | Lr per ml.

(iii) Prepare serial dilutions at intervals of 5 within the limits indicated by the above preliminary titration. Thus, if the end point is indicated as lying between 1:40 and 1:80, prepare 1:40, 1:45, 1:50, up to 1:80.

(iv) Mix 1 ml. of each dilution of toxin with 1 ml, of standard antitoxin diluted to contain 0.002 unit per ml. Incubate the mixtures for one hour at 37° C. and leave in the refrigerator overnight to allow ample time for the toxin-antitoxin reaction.

(v) Inject 0.2 ml. of each dilution mixture in-

Dilutions of	Patient's	Serum	for	Titration	Ωf	Antitovin	at	1/zooth	Level
DESCRIPTIONS OF	Pauents	Serum	IOI.		UI.	AIILILOXIII	aı	7500tH	Level

PATIENT'S SERUM (ML.)	SALINE (ML.)	DILUTION	AMOUNT MIXED WITH 1 ML, OF TOXIN DILUTION (ML.)	FRACTION OF ANTITOXIN UNIT TESTED FOR	
1.0	0	0	1	1/500	
0.5	0.5	1 in 2	1	1/250	
0.2	0.8	1 in 5	1	1/100	
0.1	0.9	1 in 10	1	1/50	
0.1	1.6	1 in 16.6	1	1/30	
0.1	2.4	1 in 25	1	1/20	
0.1	4.9	1 in 50	1	1/10	
0.1	9.9	1 in 100	1	1/5	
0.1	49.9	1 in 500	î	$\tilde{1}/\tilde{1}$	

tradermally into the shaven skin of a rabbit (as many as 40 such inoculations may be made in a single animal without ill effects), and identify the sites of inoculation with dye.

(vi) Read at 48 hours, the end point being that dilution of toxin which produces a zone of erythema nearest to 10 mm. in diameter.

Titration of antitoxin at the 1/500th level. For the titration of antitoxin in serum proceed as follows:

- (1) Dilute the stock solution of toxin as indicated in the above standardization, i.e., so that 1 ml. contains sufficient toxin to give the skin reaction when mixed with 1 ml. of antitoxin containing 0.002 unit per ml. and inoculated in amounts of 0.2 ml.
- (2) Prepare dilutions of patient's serum, say, for illustrative purposes, of 1:10 and 1:500.
- (3) Mix 1 ml. of undiluted serum and 1 ml. of each of the dilutions with 1 ml. each of the diluted toxin, and in addition set up a control which contains 1 ml. of the diluted toxin and 1 ml. of standard antitoxin diluted to contain 0.002 unit.
- (4) Incubate, inoculate, and read as above.

Suppose the toxin mixed with the 1:500 and 1:10 dilutions of serum gives a skin reaction, but that mixed with the undiluted serum does not. It follows that the undiluted serum contains more than 1/500 unit of antitoxin per ml., but it contains less than 1/50 unit per ml. since it is not neutralized by the 1:10 dilution of serum. By varying the serum dilution, any fraction of a unit may be determined as indicated in the accompanying table.

The precipitin reaction. The precipitin reaction is the specific formation of a precipitate when a soluble antigen and its homologous antiserum are mixed. It is used for the identification of soluble antigens of bacterial extracts, such as pneumococcus polysaccharide and the polysaccharide and protein antigens of the streptococci, and is used for the immunological typing of a number of kinds of bacteria. It is also used for the identification of other protein antigens

as, for instance, the identification of bloodstains in forensic medicine.

In the precipitin test antiserum is used undiluted, or in very low dilution that avoids undesirable cross reactions, and is admixed with successive dilutions of the antigen. The titer of the antiserum is expressed as the highest dilution of antigen with which precipitation occurs. It is one of the most delicate and sensitive of the serological reactions; antiserums which will precipitate an antigen such as pneumococcus polysaccharide in dilutions as high as 1:1 to 1:5 million are not uncommon, and titers to serum antigens are frequently 1:10,000 to 1:100,000. The test is carried out as follows:

(1) Preparation of the antigen dilutions

- (a) Set up a series of small test tubes, such as Wassermann tubes, each with 0.5 ml. of saline solution.
- (b) To the first tube add 0.5 ml. of the antigen solution, mix well by blowing in and out of the pipette several times, and transfer 0.5 ml. of this solution to the second tube. The procedure is continued through as many tubes as the titer, or expected titer, of the serum indicates to give a series of dilutions of the antigen to 2ⁿ. If the original antigen solution is not diluted, these dilutions will be 1:2, 1:4, 1:8, etc., but it is more convenient to prepare a 1:100 dilution of the original antigen to begin with, thus giving dilutions of 1:100, 1:200, 1:400, up to 1:25,600.

(2) Procedure of the test

- (a) Distribute the antiserum in amounts of 0.1 ml. in a series of 5 × 50 mm. test tubes, as many as the dilution range to be observed requires.
- (b) Carefully layer 0.1 ml. of the antigen dilutions onto the antiserum, each of the serial dilutions in successive tubes, so that the juncture between the two is clear and sharp.
- (c) Incubate at 37° C. for two hours, examining at 30-minute intervals for the formation of precipitate at the juncture of antiserum and antigen. This the precipitin ring test.
- (d) Shake the tubes to mix thoroughly the antiserum and antigen and store overnight in the refrigerator.
- (e) Next day the precipitate will have settled out

Protocol for the Precipitin Titration

		ANTIGEN
TUBE NUMBER	ANTISERUM (ML.)	DILUTION AMOUNT (ML.)
1 2 3 4 5 6 7 8 9 Antiserum control	0.1 0.1 0.1 0.1 0.1 0.1 0.1 0.1	1:100 0.1 1:200 0.1 1:400 0.1 1:800 0.1 1:1600 0.1 1:3200 0.1 1:6400 0.1 1:12,800 0.1 1:25,600 0.1 Saline solution 0.1
Antigen control	Saline solution 0.1	Undiluted antigen 0.1

Protocol for the Kahn Flocculation Test

TUBE NUMBER	ANTIGEN (ML.)	SERUM (ML.)	SERUM:ANTIGEN RATIO	rously	SALINE (ML.)
1	0.05	0.15	3:1	I. rogir mim	0.5
2	0.025	0.15	6:1	6 6 C	0.5
3	0.0125	0.15	12:1	nak r tv	0.5
				Sh	

and can be read by gentle agitation of the tubes.

(f) It is essential that controls of antigen plus saline, and antiserum plus saline, be included, and these should show no precipitate.

The precipitin test may also be carried out in capillary tubes to conserve antiserum, and to utilize the relatively small quantities of antigen available in bacterial extracts as in the typing of streptococci.

Flocculation tests. The so-called flocculation tests developed for the serological diagnosis of syphilis are related in a sense to the precipitin reaction, and the Kahn test is typical of these. The Kahn antigen is a lipid fraction of heart muscle soluble in alcohol but insoluble in acetone, and contains added cholesterol. When this antigen is mixed with saline in certain proportions floccules appear, and the antigen is titrated to determine the smallest amount of saline which, when added to 1.0 ml. of antigen, produces aggregates which completely disperse upon the addition of more saline. Syphilitic serum has the property of preventing this dispersion. The test is based on the mixture of constant amounts of serum with varying concentrations of antigen which are thoroughly mixed by shaking in a shaking machine, and the addition of saline. The test is set up according to the accompanying protocol and read immediately and again 10 minutes after the addition of saline. A positive reaction is indicated by the presence of floccules in the mixture.

Agglutination. Bacteria, and other particulate antigens, aggregate in clumps in the presence of homologous santiserum and are

said to be agglutinated. The agglutination reaction is useful in the serological identification of bacteria, especially those of the group of enteric bacilli such as the Salmonella and dysentery bacilli. It is also the immunological reaction made use of, in conjunction with agglutinin absorption, in the antigenic analysis of bacteria.

The agglutination reaction differs from the precipitin reaction in that the antiserum rather than the antigen is diluted, and the antibody titer of the antiserum is expressed as the highest dilution in which a constant amount of bacterial antigen is agglutinated. Agglutinating antiserums commonly show titers ranging from 1:1000 to 1:10,000 and occasionally may reach titers as high as 1:50,000 to 1:100,000, though this is rare. The agglutination of bacteria may be observed either microscopically or macroscopically; the former is generally used for typing purposes, the latter in the titration of antibody.

The microscopic agglutination test. In the microscopic agglutination test either only a few, e.g., two or three, dilutions of antiserum are used, or more commonly, a single dilution is used which preliminary titration has indicated will produce a rapid and complete agglutination with minimal cross reactions. This last allows the use of a single rapid test for typing and certain diagnostic purposes. The test is carried out as follows:

(1) Place a loopful of saline solution on a clean cover slip, and with the straight inoculating wire add

Protocol of Agglutinin Titration

	SERUM	DILUTION		AGGLUTININ TITRATION						
	SERUM (ML.)	SALINE (ML.)	DILUTION	TUBE NUMBER	SALINE (ML.)	SERUM (ML.)	ANTIGEN (ML.)	FINAL DILUTION		
A	0.1 of undiluted serum	4.9	1:50	1 2 3	0 0.25 0.40	0.50 0.25 0.10	0.5 0.5 0.5	1:100 1:200 1:500		
В	0.5 of dilution A	4.5	1:500	4 5 6	0 0.25 0.40	0.50 0.25 0.10	0.5 0.5 0.5	1:1000 1:2000 1:5000		
	0			Control	0.50	0	0.5			

a small amount of bacterial growth. The suspension should be faintly turbid, and when this point is reached, the needle is flamed and then used to stir the drop to a homogeneous turbidity.

(2) Place a loopful of diluted antiserum beside the drop of bacterial suspension. Traces of serum remaining on the loop are, of course, removed by flaming.

- (3) With the inoculating needle mix the two thoroughly. Note that this produces and additional dilution of the antiserum, and the final dilution is approximately twice the original dilution.
- (4) Mount the cover slip on a hollow ground slide with petroleum jelly as in making a hanging drop preparation. No special incubation is necessary, and in a few minutes to an hour at room temperature agglutination occurs.
- (5) The preparation may be examined under the low power objective for a curdled appearance or under high power or oil immersion objectives for direct observation of the agglutinated bacteria. False clumping may occur and is distinguished from true agglutination in that in the latter all of the bacteria are gathered in large clumps with none about the edges of the drop, but false clumping occurs in small aggregates about foreign particles and around the edge of the drop.

Macroscopic agglutination. The titration of agglutinin is carried out in a manner similar to the titration of precipitin except, as noted above, with varying amounts of serum in the presence of a constant amount of antigen.

- (1) PREPARATION OF ANTIGEN
 - (a) Inoculate an agar slant culture with the bacteria to be used as antigen, spreading the inoculum over the entire surface of the slant.
 - (b) After 18 hours' incubation run about 2 ml. of saline solution on the agar slant culture. The saline may contain 0.5 per cent formalin if a formalinized antigen is to be used.
 - (c) Rub up the bacterial growth with an inoculating needle or loop to give a uniform suspension of the bacteria in the saline.
 - (d) With flamed forceps remove a small piece of cotton from the bottom (sterile) part of the cotton plug of the culture tube and drop it into the saline suspension.
 - (e) With a sterile pipette push the piece of cotton to the bottom of the saline, taking care to leave the cotton over the pipette opening.
 - (f) Draw up the bacterial suspension into the pipette through the cotton, which serves to

filter out coarse particles, and add this suspension slowly to the proper turbidity to whatever volume of saline solution is required for the titrations to be undertaken. (A single agar slant culture will make 100 to 300 ml. of antigen, depending on the amount of bacterial growth and the density of the final antigen.) The turbidity of the final antigen such that, when diluted with an equal volume of saline, it will be lightly turbid in the test tubes used for the titration. The precise turbidity is dependent upon the kind of antigen and antibody and on the personal preference of the worker.

- (2) PREPARATION OF SERUM DILUTIONS
 - (a) Serum may be diluted in essentially the same manner as antigen is diluted in the precipitin test, and the dilution is carried out in 10×75 mm. test tubes used for the titration of agglutinin since the antigen is added directly to the diluted serum. A common procedure is to set up a series of saline blanks, the first containing 0.9 ml. and the remainder 0.5 ml. The first dilution is 1:10, made by adding 0.1 ml. of serum to the 0.9 ml. blank; the second and subsequent dilutions are made by transferring 0.5 ml. portions of each preceding dilution. This gives dilutions of 2^n , i.e., 1:10, 1:20, 1:40, . . . etc.
 - (b) The above method is the one of choice when only a very few titrations are to be run but is laborious and time-consuming if there are many titrations. An alternative, more efficient procedure less subject to dilution error arising from carrying over excess serum in the pipette, since so few pipettes are used that a fresh one for each dilution is practical, is shown in the above table. In this method only one direct dilution is made for each three final serum dilutions, and the dilutions closely approximate 2ⁿ.
- (3) PROCEDURE OF THE TEST
 - (a) To each of the series of tubes containing 0.5 ml. each of the several serum dilutions, add 0.5 ml. of antigen suspension and mix by gentle agitation. Note that this doubles the serum dilution, and the final dilutions are 1:20, 1:40, 1:80, . . . etc., when made by the first method; the final dilutions in the second method are given in the table.
 - (b) The method of incubation is variable, depending

upon the particular system being titrated, and the following methods are common:

- (i) Incubate at 37° or 55° C. for two hours, read and store overnight at room temperature or in the refrigerator, and read again.
- (ii) Incubate at 37° C. overnight and read the following morning.
- (c) The agglutinated bacteria form clumps of precipitate in the bottom of the tubes and are read in a cross light to give a Tyndall effect by gently agitating the tubes individually to swirl the agglutinated bacteria up into the supernatant. The character of the agglutinated bacteria differs in different circumstances; the H agglutination of Salmonella. for instance, is flocculent, while the O agglutination is finely granular. Some workers record degrees of agglutination from + or barely perceptible partial agglutination to ++++ of complete agglutination with a clear supernatant, while others simply record the presence or absence of agglutination with a single + or - sign. The agglutinin titer of the antiserum is the highest dilution in which definite agglutination occurs.

Complement fixation. The complement fixation test is based on the observation that complement, a heat-labile constituent of normal serum, combines with an antigenantibody complex and is said to be fixed. Since this fixation produces no visible change, a hemolytic system, consisting of sheep erythrocytes and anti-sheep cell hemolysin, is added. If the complement is free, i.e., has not been fixed, and inferentially the original union of antigen and antibody has not occurred, the erythrocyte-hemolysin complex combines with the complement. and visible lysis of the red cells occurs. Conversely, if the erythrocytes are not lysed, the test antigen-antibody system has combined and fixed the complement, leaving none for hemolysis. This complement fixation test is most often applied in the serodiagnosis of syphilis and virus and rickettsial infections. Bacterial antigens fix complement readily.

The following reagents are required:

- (1) The antigen-antibody system to be tested, either component of which may be unknown.
- (2) Anti-sheep erythrocyte hemolysin, prepared by the immunization of rabbits with sheep red cells.
- (3) A 2 per cent (by volume of packed cells following centrifugation) suspension of washed sheep erythrocytes in saline.
- (4) A source of complement, practically always fresh guinea pig serum.

Titration of reagents. Since the test is necessarily quantitative with respect to the relative proportions of the various components, the activity of these must be titrated prior to the actual test. These preliminary titrations include:

- (1) The titration of hemolysin which is carried out by varying the amount of hemolysin in the presence of constant amounts of sheep erythrocytes and complement, the last in amounts more than necessary for complete lysis. This titration is carried out as indicated in the accompanying protocol. The smallest amount, i.e., highest dilution, of hemolysin required to bring about complete lysis is the hemolytic unit. In the final test two units in a volume of 0.5 ml. are used in each tube and on the basis of this titration the hemolysin is diluted to contain four units per ml.
- (2) The complement of fresh guinea pig serum is similarly titrated, using variable amounts of complement in combination with constant amounts of erythrocytes and hemolysin as shown in the accompanying protocol, and the unit of complement is defined. The "exact unit" is the smallest amount that gives complete hemolysis, and the next larger amount, i.e., in the adjacent tube, is the "full unit." In the complement fixation test two full units in a volume of 1.0 ml. are used in each tube. The reason for the excess amount is

Protocol of Hemolysin Titration

TUBE NUMBER	HEMOLYSIN DILUTION (0.5 ML.)	COMPLEMENT 1:30 DILUTION (ML.)	2% suspension erythrocytes (ML.)	SALINE (ML.)
	1:1000	0.3	0.5	1.7
$\frac{1}{2}$	1:2000	0.3	0.5	1.7
3	1:3000	0.3	0.5	1.7
4	1:4000	0.3	0.5	1.7
5	1:5000	0.3	0.5	1.7
6	1:6000	0.3	0.5	1.7
7	1:8000	0.3	0.5	1.7
8	1:10,000	0.3	0.5	1.7
Control (9)*	1:1000	None	0.5	2.0
Control (10)*	None	0.3	0.5	2.2

^{*}Both controls must be negative.

Protocol of Complement Titration

TUBE NUMBER	1:30 dilution complement (ML.)	SALINE (ML.)	HEMOLYSIN 4 UNITS/ML. (ML.)	2% suspension erythrocytes (ml.)	
1	0.20	1.8	0.5	0.5	
2	0.25	1.8	0.5	0.5	
3	0.30	1.7	0.5	0.5	
4	0.35	1.7	0.5	0.5	
5	0.40	1.6	0.5	0.5	
6	0.45	1.6	0.5	0.5	
7	0.50	1.5	0.5	0.5	
Control (8)*	0.00	2.5	0.0	0.5	

^{*}Control must be negative.

that some complementary activity is lost during the time allowed for fixation; even if no fixation occurs, the test is sufficiently sensitive even with this slight excess.

(3) Certain properties of the antigen are pertinent to the test and must also be determined by preliminary titration. These properties include anticomplementary activity, i.e., inhibition of the action of complement, hemolytic activity, and binding power for complement. The titration of these is shown in the accompanying protocol. In general, the binding power of the antigen should be at least 10 times its anticomplementary action, and in the final test not more than one third of the amount of antigen found to be anticomplementary may be

used. It is self-evident that the antigen must not lyse red cells in the concentrations used in the test.

Procedure of the test

- (1) Inactivate the test serum, i.e., destroy any traces of of complement in it, by heating to 56° C. for 15 to 20 minutes; dilute 1:5 and distribute in three 15 × 100 mm. test tubes as indicated in the accompanying protocol. Bring the total volume in tubes 2 and 3 to 0.5 ml. by adding 0.25 and 0.375 ml. of saline respectively.
- (2) Add 0.5 ml. of antigen, diluted to contain 20 complement-fixing units per ml. to each tube.
- (3) Let the mixture stand at room temperature for 10 minutes.

Protocol of Antigen Titration

PROPERTY TESTED	TUBE NUMBER	ANTIGEN* (ML.)	ANTISERUM* 1:25 DILUTION (ML.)	COMPLE- MENT 1:10 DILUTION (ML.)	SALINE (ML.)		HEMOLYSIS 4 UNITS/ML. (ML.)	% SUS- PENSION ERYTHRO- CYTES (ML.)
Anticomplemen- tary activity	1 2 3 4 5 6	0.5 0.4 0.3 0.2 0.1 0.05	0 0 0 0 0	0.3 0.3 0.3 0.3 0.3 0.3	0.2 0.3 0.4 0.5 0.6 0.65	refrigerator overnight	0.5 0.5 0.5 0.5 0.5 0.5	0.5 0.5 0.5 0.5 0.5 0.5
Hemolytic ac- tivity	7 8	0.5 0.1	0	1.0 0	1.0 1.4	.⊑	0 0	0.5 0.5
Complement- binding power	9 10 11 12 13 14	0.5 0.25 0.1 0.075 0.05 0.025	0.25 0.25 0.25 0.25 0.25 0.25	0.3 0.3 0.3 0.3 0.3 0.3	0.05 0.2 0.35 0.375 0.4 0.425	for one hour or store	0.5 0.5 0.5 0.5 0.5 0.5	0.5 0.5 0.5 0.5 0.5 0.5
Controls	15† 16‡	0 0	0.25 0.25	0.3	0.45 1.75	37° C. fa	0.5 0	0.5 0.5

^{*}The amounts of antigen and antiserum given here are arbitrary and will vary with individual preparations.

[†]This control should show complete hemolysis.

[‡]This control should show no hemolysis.

Protocol of the Complement-Fixation Test*

TUBE NUM- BER	SERUM DILUTED 1:5 (ML.)	ANTIGEN 20 UNITS/ML. (ML.)	SALINE (ML.)		COMPLE- MENT 2 UNITS/ML. (ML.)		HEMOLYSIN 4 UNITS/ML. (ML.)	2% suspension erythrocytes (ML.)
1 2 3 4 (SC) 5 (AC) 6 (HC) 7 (EC)	0.5 0.25 0.125 0.5 0 0	0.5 0.5 0.5 0 0.5 0	0 0.25 0.375 0.5 0.5 1.0 2.5	Let stand at room tem- perature for 10 minutes	1.0 1.0 1.0 1.0 1.0 1.0	37° C. for one hour, or refriger- ator over- night and 5–10 min- utes 37° C.	0.5 0.5 0.5 0.5 0.5 0.5	0.5 0.5 0.5 0.5 0.5 0.5 0.5

*Set up as the Kolmer modification of the Wassermann test.

SC serum control-should show complete hemolysis.

AC antigen control—should give complete hemolysis.

HC hemolytic system control—should give complete hemolysis.

EC erythrocyte control—should give no hemolysis.

- (4) Add 1.0 ml. of complement, diluted to contain two full units per ml., to each tube.
- (5) The mixture may be incubated in a water bath at 37° C. for one hour, or stored in the refrigerator overnight and warmed in the water bath for 10 to 15 minutes before proceeding with the test the following day. This time interval is allowed for fixation of the complement by the antigen-antibody complex, and the later method is referred to as "icebox fixation."
- (6) Add 0.5 ml. of hemolysin diluted to contain four hemolytic units per ml. to each tube.
- (7) Add 0.5 ml. of a 2 per cent suspension of washed sheep erythrocytes to each tube.
- (8) The appropriate controls of the serum, antigen, hemolytic system, and erythrocyte suspension are indicated in the protocol and must be included.
- (9) Incubate in a water bath at 37° C. for 15 to 60 minutes.
- (10) Read the hemolysis as complete (++++), partial (+++, ++, +, ±), or negative.

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Chapter Three

THE PHYSICAL AND CHEMICAL STRUCTURE OF MICROORGANISMS

One of the most striking aspects of the morphological consideration of microorganisms is their extremely small size. This order of size is such that they are most conveniently measured in the units of the micron $(\mu = 10^{-3} \text{ millimeters})$ and the millimicron $(m\mu = 10^{-6})$ millimeters). The proper, those capable of independent metabolic existence in that they are cultivable on lifeless nutrient mediums, range in size from that of the large bacilli,* such as Bacillus anthracis, with dimensions of 1.0 to 1.3 \times 3 to 10 μ , to minute forms such as Pasteurella tularensis measuring 0.2×0.2 to 0.7μ and microorganisms of the pleuropneumonia group in which viable elements as small as 175 m μ are demonstrable.

The rickettsiae and viruses, characterized by their inability to proliferate apart from a living host cell, form a continuous series of even smaller forms, running through and beyond the limits of optical resolution, commonly taken to be 200 mµ but in practice about 250 m μ . The rickettsiae resemble the small bacteria, having dimensions of 0.2×0.5 to 1.5 μ , and the poxviruses, whose elements measure 200 to 350 m μ , also overlap the smallest bacteria. From this maximum, the viral agents range down to nearly macromolecular dimensions such as 25 m μ for poliomyelitis virus and 22 m μ for foot-and-mouth disease virus, while the egg albumin molecule, for example, measures 2.5×10 m μ . The relative size of representative micoorganisms is illustrated in Figure 3.

The range in size among individual bacterial cells of the same species differs from one species to another, being greatest in the bacillary forms and least in the spherical types. Excluding filamentous and swollen forms that may be produced under appropriate conditions and are usually regarded as abnormal, the maximum difference in size between mature individual cells is about fourfold to fivefold.

The structure and physiological processes of a living organism are intimately associated with, and in fact are a function of, its size. 109 While substantially the same mechanisms are functional in terrestrial mammals from the elephant (400 kg.) to the pigmy shrew (4 gm.), a mammal smaller than the very small rodents cannot exist because it could not assimilate and metabolize nutriment rapidly enough to supply the energy it requires. Similarly, the rate of oxygen diffusion in insect tracheas limits the size range of organisms using this respiratory mechanism between a maximum represented by some tropical forms 5 to 8 cm. in length and the minimum of small mites 0.2 mm. long.

The lymphocyte, one of the smallest mammalian cells, with a diameter of 10μ and a volume of $524 \mu^3$, is many times larger than B. anthracis with a maximum volume of $22 \mu^3$, and the minimum volume of $0.004 \mu^3$ of Past. tularensis. The last appears to represent the minimum size that allows the

^{*}Excluding the often quoted but exceptional case of *Bacillus biitschlii*, found in the cockroach intestine, and reported to be as large as $0.6 \times 80 \mu$.

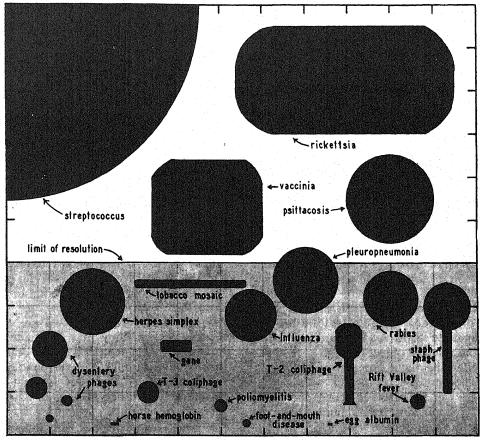


Figure 3. Diagrammatic representation of the relative size of representative microorganisms. The pips are at intervals corresponding to 100μ , and the small squares of the grid to $10~m\mu$. The microorganisms in the shaded portion are those whose size is below the limits of resolution of the light microscope.

degree of organization necessary to independent metabolism in that the smaller agents, the viruses, are obligate intracellular parasites.

The size range of the obligate parasites overlaps that of the metabolically independent microorganisms in that the larger forms, such as Coxiella burnetii having a volume of $0.02 \ \mu^3$ and the largest viruses, such as vaccinia virus with a volume of $0.008 \ \mu^3$, are larger than Past. tularensis and other extremely small bacteria. But smaller forms, running in continuous series to the apparent minimum of the volume of $0.00005 \ \mu^3$ of foot-and-mouth disease virus, can at best contain only relatively few protein molecules.

While metabolic processes on a molecular level need not differ significantly between large and small physiological units, it may be anticipated that the relative lack of cellular structure may result in unique characteristics of the minute forms. This has proved to be true, in part because these minute forms do not appear to be closely restricted to the hereditary mechanisms, processes of proliferation, etc., to which the more elaborately organized unicellular and multicellular organisms are limited, and in part because of the special circumstances of their macromolecular environment. The uniqueness of the microorganisms is more apparent than real, then, in that their unusual properties and behavior represent an extension of, rather than a contrast with, the patterns of basic biological phenomena.

MICROSCOPY24

A consideration of the technical limitations and advantages of the various methods available for the demonstration of the shape and structure of microorganisms is an essential preliminary to a consideration of the morphology of these forms. In general, these methods are of two kinds, *viz.*, those utilizing light, both visible and ultraviolet, and that making use of a focused beam of electrons.

compound microscope. With The ordinary microscope, objects are viewed by transmitted light, i.e., the object is interposed between the source of light and the eve or photographic plate. In order to be seen and delineated (resolved) the object must be sufficiently large to cast a shadow, and the critical size is approximately half the wave length of the light used. The highest numerical aperture that is practical in the objective lens is 1.4, and this permits resolution of objects down to the critical size. The limit of resolution is obviously a function of the wave length of light used. Thus in the range of 5000 Å (500 m μ), or blue light, to 5500 Å (550 m μ), or yellow light, the smallest object that can be resolved is a sphere having a diameter of 250 to 275 mu. Theoretically an object as small as 200 m μ can be resolved using deep blue light at 400 m μ wave length, but the practical limit is less than this.

Resolving power may be appreciably extended by the use of light in the ultraviolet region; assuming a wave length of 250 m μ , a sphere 125 m μ in diameter may be resolved. Quartz lenses are, of course, required.

The darkfield microscope. In the darkfield microscope reflected rather than transmitted light is used, taking advantage of the familiar Tyndall effect perhaps most commonly seen in the appearance of particles of dust in a shaft of sunlight in a darkened room. The usual compound microscope may be used by substituting a darkfield condenser, which lights the object from the side, for the ordinary substage condenser. Relatively large amounts of light are required, and oil is placed between the top of the condenser and the slide to reduce loss at these interfaces.

The object appears brilliantly lighted against a dark background. There is an illusion of increased resolution in that extremely small objects appear as bright points of light in contrast with the minute shadows cast in transmitted light, but, in fact, resolution is not increased beyond the critical limits noted above. Thus the darkfield micro-

scope has not contributed appreciably to bacterial cytology other than with regard to certain aspects of the activity of flagella. It has, however, very considerable utility in the demonstration of very slender microorganisms, especially spirochetes, as in exudate from a syphilitic chancre or leptospira in the blood from cases of leptospirosis.

The phase microscope. 9, 83 Light passing through an object of different thickness or refractive index from that of the surrounding fluid is scattered, with the result that it is out of phase and reduced in amplitude, i.e., the distance from the crest to the trough of the light wave is reduced. Differences in amplitude are visible and allow the observation of the object as a relatively faint shadow against its background, and bacteria may be so observed in the living unstained state, usually in a wet preparation.

While differences in phase are not ordinarily visible, they may be made so by treating the light scattered by the object and that coming through the surrounding fluid independently so that they interact, either to interfere with one another to darken the image or to reinforce one another to brighten it. This is accomplished with the ordinary microscope by restricting the incident light to an annular beam by means of an annular diaphragm at the lower focal plane of the condenser and the insertion of a corresponding annulus or diffraction plate at the back focal plane of the objective lens. The microscope so modified is a phase microscope and may be either bright or dark phase.

Phase contrast microscopy has been particularly useful in the study of structures in the living cell, unmodified by the artifacts inherent in the processes of fixing and staining. By increasing contrast, it gives greatly improved resolution, for example, allowing the demonstration of bacteria in unstained tissue sections that are not resolved by the bright- or darkfield microscopes because of the relatively great diffraction and reflection of the tissue elements. The phase microscope is, however, subject to the same limits in ultimate resolving power as other forms of the microscope using visible or ultraviolet light.

The electron microscope. 49, 120 The electron microscope operates on a different principle from that of the optical microscopes in that a stream of electrons is used rather than a beam of light. The electron

beam is focused by magnetic "lenses" and the interposed object intercepts the beam to cast a shadow which is recorded on a photographic plate to give an electron micrograph. The theoretical resolving power of the electron beam is about 100,000 times that of visible light, but this limit is not reached in practice because of defects in the magnetic focusing corresponding to chromatic and spherical aberration in optical systems. Nevertheless, remarkable resolution is possible at 10,000 to 50,000 diameters and, because of the small aperture used, the depth of focus is considerably greater than that obtainable with the 1.4 mm. objective.

The technique of shadow-casting with heavy metals vaporized under high vacuum and deposited at an angle on the preparation was developed by Williams and Wyckoff in 1946. The metal film is opaque to electrons, and the resulting electron micrograph has a brilliantly lighted three-dimensional

appearance. Subsequently the technique of negative staining with phosphotungstate, analogous to the negative staining techniques of light microscopy, was introduced. It has been particularly useful in the elucidation of the structure of virus particles since it shows fine structures not apparent in thin sections of particles embedded in osmic acid-methylacrylate.

Application of electron microscopy to microorganisms has made it possible to see details of structures lying at the limits of optical resolution and to determine directly the size and shape of small viruses previously studied only by indirect methods such as sedimentation and filtration. The results of electron microscopy must be interpreted with some caution because specimens can be examined only as dead dry material because of the high vacuum required and are subject to the very considerable distortion of drying.

The Morphology of Bacteria 14, 105, 107

The morphology of bacteria may be considered under two general heads: that of individual cells and groups of cells as observed under high magnifications, and that of bacterial colonies developing on solid mediums and consisting of very large numbers of cells that are visible to the naked eye. In the first instance differences in shape and certain structural details as well as size are characteristic of at least the main groups of bacteria and provide the primary basis for their systematic study. Similarly, the masses of cells represented in the bacterial colony also have characteristics such as size, consistency, texture, and color that have differential value though not the fundamental significance of cellular morphology.

THE BACTERIAL CELL

Shape. The most obvious difference among bacteria is their shape, and three general morphological types are clearly evident. These are the spherical form or coccus, the rod-shaped form or bacillus, and the spiral forms, the various subtypes of which are the vibrio, spirillum, and spirochete.

Coccus. The spherical bacteria are by

far the most homogeneous of the bacteria with respect to size and, in general, have a diameter of 0.8 to 1.0 μ , although smaller varieties, 0.4 to 0.8 μ in diameter, have been described. There is some small deviation from the spherical form among bacteria grouped as cocci. The pneumococcus, for example, has a lanceolate shape, slightly elongated with one end more pointed than the other, and the gonococcus and meningococcus tend to a coffee bean shape. Other slightly elongated forms are referred to as coccobacilli, and the line of demarcation is not precise.

Morphological subtypes of the cocci are differentiated on the basis of the arrangement of the individual cells with respect to one another. This is a consequence of two factors, one the plane or planes in which cell division occurs, and the other the tendency of daughter cells to remain superficially attached or close to one another after division is complete.³⁹

Those cocci which separate completely after cell division, regardless of the plane of division, appear singly, and scattered at random over the microscopic field are called *micrococcus*. When there is a slight tendency for daughter cells to remain attached, and cell division occurs in only one plane, the

cocci tend to occur as pairs, diplococcus, but the paired cells are intermingled with individual cells. Some kinds of diplococci are slightly elongated and are paired with the long axes parallel. In the case of the coffee bean-shaped gonococcus and meningococcus noted before, the paired cells have the convex sides adjacent. When the tendency to remain attached is more marked, the result is chains of cells, streptococcus, consisting of four to ten or a dozen cells that often have the appearance of diplococci attached end to end.

When there is a marked tendency of daughter cells to remain adjacent to one another coupled with cell division in two or more planes, the cocci occur as irregular groups, staphylococcus, which are often quite large. When observed in the usual stained preparation, staphylococci appear to consist of sheets of cells, i.e., only one cell thick, but on examination in a wet mount are seen to consist of irregular, grape-like clusters. The former would result from cell division occurring in only two planes, and the latter from division in three planes, but it is probable that the sheets of cells are, for the most part, an artifact resulting from the preparation of the smear. When the tendency to remain together is less marked, the cocci are found in tetrads or groups of four cells and in cubical packets of eight cells. There is no casual name for these smaller aggregates, but they are characteristic of some genera, such as Sarcina.

Separation of these subtypes of coccus on a morphological basis is not completely sharp. Thus, diplococci occur admixed with single cells, streptococcus chains are interspersed with paired and single cells, and single and paired cells, as well as an occasional chain of four cocci, are found in stained smears of staphylococci. It is obvious that these groupings can be broken up to a greater or lesser extent in preparing the bacterial suspension for examination and that the occurrence of typical groupings is not absolute, but the consequence of only a tendency to remain adjacent. In practice, though, the characteristic morphological groups are usually found without difficulty and are most consistently observed among the staphylococci.

Bacillus. The cocci merge into the rodshaped bacteria through connecting links such as the lanceolate coccus and coccobacillus noted above. In turn, the rod-shaped or bacillary form is a collective term that embraces a variety of morphological subtypes.

The morphology of individual rod-shaped bacteria differs considerably between genera and even species. Not only is there variation in size, but the shape of the individual cell differs, sometimes markedly. Some are long and slender while others may be short and thick; the sides may be more or less parallel to one another, or the cell may be thicker in the center and taper toward the ends to give the form known as the fusiform bacillus.



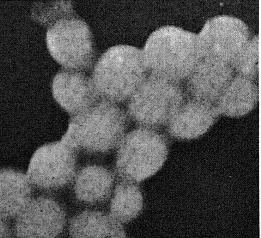


Figure 4. Electron micrographs of gold-shadow-cast preparations of *Bacillus subtilis* and *Staphylococcus aureus*. The shadow-cast preparation illustrates particularly well the occurrence of staphylococci in clusters of attached cells. The cap at the ends of the bacillus and a single flagellum are shown. (Lilly Research Laboratories.)

SPIROCHETES 41

Similarly, the ends of the cells may be square and cut off sharply, or even give the appearance of a slight concavity, while the ends of other cells are rounded. These variables can occur in a number of combinations to give a considerable heterogeneity in the bacillary form. A given shape is, however, relatively constant within a species under standard conditions of growth, though the length-width ratio may vary, owing in large part to elongation of individual cells prior to cell division.

Like the cocci, some of the bacillary forms assume characteristic groupings, but these are a consequence of postfission movements, for the plane varies rarely if at all from that vertical to the long axis. There is a tendency of the cells to remain attached, or closely adjacent, after cell division in some kinds of bacilli; it is marked in some forms, notably the spore-forming aerobic organisms of the genus Bacillus, and may be enhanced under appropriate conditions of growth. When this occurs, it results in the formation of chains of cells, the *streptobacillus* morphological type.

The postfission movements of bacilli are of two general kinds. The one, known as slipping, consists of a sliding movement of the cells against one another with the long axes parallel to give groups of cells with a palisade-like appearance. This appears to be a consequence of space limitations interfering with continued extension of growth in a longitudinal axis. In the other kind of movement, snapping, after division the daughter cells bend sharply with respect to one another to an acute angle to give a V-shaped appearance. When this kind of movement is associated with a tendency to remain attached end to end, chains of cells resembling a split-rail fence result.40

While to a certain extent characteristic, the groupings assumed by the rod-shaped bacteria have only minor differential significance in contrast to the cocci among which they make possible morphological subtypes of generic status.

The spiral forms. The third main morphological type of bacteria is made up of the spiral forms which may be regarded as bacillary forms twisted in the form of a helix. Again, forms intermediate on a morphological, though not necessarily phylogenetic, basis occur as the curved rods. These range from the occasionally curved

coliform bacilli to those forms whose curvature is sufficiently consistent to have differential significance in the definition of the genus Vibrio. Whether the vibrios are truly transitional forms linking the slender bacilli with the spiral microorganisms is questionable, but their occasional end-to-end grouping in which the direction of curvature of the individual cells alternates superficially resembles the spiral form.

The spiral bacteria, which if straightened out would be long slender bacilli, are of two general kinds, the one in which the spiral is rigid, the genus Spirillum, and the other in which it is flexible. Among the latter, a further differentiation is made on the tightness of the coiling. The tightly coiled forms include several genera which are differentiated by finer morphological criteria. These include differentiation of an outer sheath, a central filament about which the protoplasm is coiled, etc.; such structural details are illustrated in the accompanying electron micrograph.

One of these is the genus Treponema which includes pathogenic forms, notably the causative agent of syphilis and related diseases, together with a wide variety of nonpathogenic but parasitic forms such as those making up a part of the normal flora of the mouth. Still another form, distin-

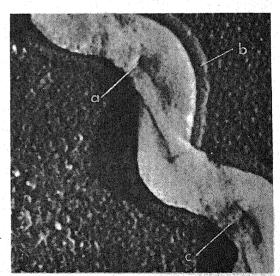


Figure 5. Electron micrograph of a chromium-shadow-cast preparation of Leptospira icterohaem-orrhagiae. The axial filament is separated from the protoplasmic spiral by a narrow space at (a) and is more closely approximated at (c). The sheath is shown at $(b) \times 125,000$. (Simpson and White.⁹²)

guished by sharp, hook-like bends at the ends of the cell, makes up the genus Leptospira, a causative agent of infectious jaundice.

Much less tightly coiled spiral forms which have, in fact, the appearance of extremely long, slender, undulating bacillary forms, make up the third morphological subtype. This includes both parasitic and pathogenic forms, the latter causing the louse- and tick-borne relapsing fevers in man and related diseases in certain fowl, and they are grouped under the genus Borrelia.

Taken together, the spiral forms are casually referred to as the spirochetes. The emphasis on morphological criteria for purposes of differentiation and definition of genera is a consequence of their failure, with the exception of Leptospira, to grow on laboratory mediums and, therefore, a complete ignorance of differential physiological activity that provides much of the basis for the characterization of other bacteria.

Involution forms. The great majority of bacteria are relatively constant in shape and size in young cultures actively growing under favorable conditions. In older cul-

tures in which many of the cells are dead or dying, the cell structure breaks down and aberrant forms appear. These include balloon-like structures, Y-shaped bacillary forms, cells containing large amounts of granular material, and the like. These are degenerative forms or involution forms and presumably result from breakdown of the mechanisms of selective permeability with imbibition of water, autolysis of cell structures by the action of proteolytic and other intracellular enzymes, etc. Some workers have attached significance to bizarre forms found in older cultures, regarding them as representative of an orderly sequence of morphological types indicative of a complex life cycle.

Aberrant forms of bacteria may also be p oduced by growing them under somewhat adverse conditions, such as higher than optimum temperatures, in the presence of relatively large concentrations of inorganic salts,³⁷ and sublethal concentrations of antibacterial substances. In the case of the last, the antibacterial activity may be largely growth-inhibiting rather than bactericidal, one result of which may be an inhibition of



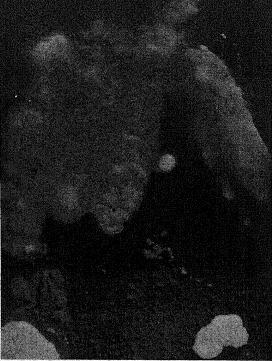


Figure 6. Electron micrographs of gold-shadow-cast *Bacterium coli* from old cultures. One intact cell is present, and the remainder show the granulation of dead and disintegrating cells. (Lilly Research Laboratories.)

the processes of cell division rather than of synthesis of cell substance, so that giant cocci, etc., develop in such cultures.

THE STRUCTURE OF BACTERIAL CELLS

The total physiological activity of the bacteria is, in many respects, so closely similar to that of the larger forms, that the assumption of at least some common mechanisms on a molecular or macromolecular level is more than a working hypothesis. While it is obvious from even cursory examination that the bacterial cell is discontinuous and therefore has structure, at the same time considerations of size make it equally clear that its structural elements need not be, and in fact in some instances perhaps cannot be, small scale replicas of those found in larger cells. The problem becomes, then, one of elucidation of the nature of subcellular elements and of their relation to the physiological functions of the cell.

The reference point is necessarily the living cell, and the information obtained by direct observation is subject to the limits imposed by the degree of optical resolution possible with visible, or ultraviolet, light. This basic information is supplemented and complemented by studies on killed cells treated with stains and other cytochemical reagents and/or subjected to observation

under the high magnifications obtainable in the electron microscope. Such supplementary information must be treated with due reservation because of artifacts resulting from fixing, drying, and other preparative procedures.

For purposes of discussion the bacterial cell structures may be divided into two groups, those structures which are external to the cell proper and those which occur within the cell.

EXTERNAL STRUCTURES

Two kinds of structures are external to the bacterial cells, the flagella or organs of locomotion and the capsular material or slime layer surrounding the cell. Neither is essential to the continued existence of the cell. A bacterium may be deprived of its flagella, as by mechanical shaking, or of its capsule by enzymatic digestion, without inhibition of growth or metabolic functions.

Flagella. $^{56, 101, 116}$ The bacterial flagellum is a long filamentous appendage originating in a spherical body, or basal granule, about $100 \text{ m}\mu$ in diameter and located in or just inside the cell wall. It is not clear whether these bodies correspond to the blepharoplasts of ciliated protozoa, but flagella are distinct from, though analogous to, cilia.

The flagellum is usually 12 to 15 m μ in diameter, without taper, and of variable

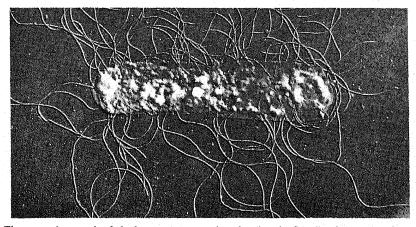


Figure 7. Electron micrograph of shadow-cast preparation showing the flagella of *Proteus vulgaris*. The bacteria were grown to the swarming stage on agar, refrigerated at 5° C. for 16 to 20 hours, floated off in 5 per cent formalin, and washed twice before mounting on collodioh. The preparation is very transparent and flattens completely so that certain of the internal structures are demonstrable by shadowing. The origin of the flagella in small, spherical bodies about 100 m μ in diameter and remarkably uniform in size is clearly shown. (van Iterson.)

length, in an extreme range of 1 to 70 μ , but ordinarily 15 to 25 μ . In the living cell it is coiled in the form of a cylindrical helix, but in dried preparations the third dimension is minimized and it appears as a wavy filament. Because of its length the flagellum is demonstrable occasionally in the living cell by phase contrast microscopy and more readily with the darkfield microscope. In mordanted stained preparations, the stain is deposited on the flagellum to make it sufficiently thick that it may be observed with the conventional microscope and is, of course, readily seen in electron micrographs.

Flagella occur most commonly, although not exclusively, among the rod-shaped bacteria. They may be attached either at or near the poles of the cell or distributed over the rest of the cell surface with a tendency for the poles to remain bare, and their location and number are relatively constant within bacterial species. The classification of bacteria with respect to the occurrence of flagella is the early one of Messea. According to this, the bacteria having a single polar flagellum are designated monotrichous. those having two or more flagella at one end of the cell lophotrichous, those having tufts of flagella at both ends of the cell amphitrichous, and those having flagella, usually eight to 12, distributed over the cell surface are peritrichous.56

The rate of movement of motile bacteria is often surprisingly rapid. For example, the motile enteric bacilli, such as the typhoid, coliform, and related bacteria, have been found to average speeds of 25 to 30 μ per

second and the cholera vibrio to move as fast as 55 μ per second. These rates are comparable with automobile speeds of 40 to 80 miles per hour.

The flagellar substance is antigenically distinct from that of the cell, and antibody to it is specific. The function of flagella in motility is seriously interfered with in the presence of specific antibody, and the organisms are immobilized because of agglutination of the flagella.⁶⁷ This is the basis of the "immobilization test" which has considerable serodiagnostic utility in diseases such as syphilis.

Flagella also differ in chemical composition from the cell substance proper. They may be removed from the cell by vigorous shaking and separated by differential centrifugation. They are built up as linear polymers of small protein molecules which are depolymerized in weak acid (0.05 M CHI) to give a clear colorless solution and an acidinsoluble fraction rich in phosphorus and carbohydrate. The protein may be repolymerized in strong salt (e.g., 50-60 per cent $(NH_2)_2$ SO₄) solutions to give an opaque viscous solution. In this form it behaves like a solution of long thin protein molecules, and is found in electron micrographs to consist of small rod-like particles resembling fragmented flagella. Its amino acid composition is characteristic, and no histidine, tryptophan, hydroxyproline, or cystine is found in detectable amounts. The chemical composition appears to be the same in different genera of bacteria such as Proteus and Bacillus.

X-ray diffraction studies have shown

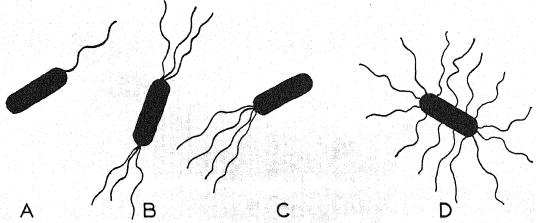


Figure 8. Position of flagella on the bacterial cell. A, monotrichous; B, amphitrichous; C, lophotrichous; D, peritrichous.

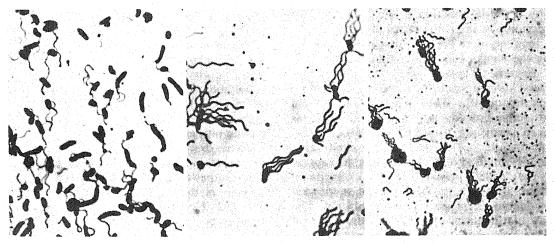


Figure 9. Flagella of bacteria in stained smears examined with the optical microscope. Left, single polar flagellum of a water vibrio; center, peritrichous flagella of Sal. typhimurium; right, peritrichous flagella of Rhizobium leguminosarum. × 2000. (Kral.)

that the flagellar protein, for which the name flagellin has been suggested, is similar to keratin, myosin, epidermin, and fibrinogen. Two patterns are observed, the one suggesting polypeptide chains folded in the α -configuration, and the other the cross- β -configuration regarded as characteristic of actively contracted muscle. This has been taken to imply that the motion of flagella attached to the living cell is a result of rhythmic interchanges between the two configurations. 6

The motion of flagella is apparently random; fibrous internal conducting structures found in ciliates seem to have no counterpart in the motile bacteria, and such a "primitive nervous system" is lacking. The seemingly coordinated motion of flagella is a hydrodynamic phenomenon, occurring when the random flagellar motions of a multiflagellar bacterium coincide by chance to initiate movement of the cell. 17, 47, 77

The energy expenditure required for motility has been calculated⁷³ to be about 14 electron volts per second, or, assuming a 25 per cent efficiency, a total output of 56 electron volts per second. Further, the analogy between muscle fibers and the observed contraction of isolated flagella in the presence of ATP has been taken to suggest that the source of energy is that liberated in the metabolism of energy-rich phosphate bonds, with about 150 bonds reacting per second. Since each flagellum flicks 10 to 20 times per second, and assuming 10 to 20 flagella on a peritrichous bacterium, it is possible

that each flick is the consequence of a discrete metabolic event, such as the breakdown of an energy-rich phosphate bond.

Fimbriae. In a study of bacterial flagellation. Houwink and van Iterson⁴⁵ described minute filamentous appendages on some kinds of flagellated bacteria which have subsequently been referred to as pili, bristles, filaments, and fimbriae; the last appears to have gained some general acceptance. Studies by others¹⁵ have shown that fimbriae are less rigid than flagella, and considerably smaller, 10×300 to 1000 m μ , 100 to 250 per bacterial cell. In the case of dysentery bacilli they appear to facilitate adherence of the bacteria to the intestinal mucosa and, in the Flexner dysentery bacilli, contain an antigen common to the several serotypes (Chap. Twenty-two). Not all flagellated bacteria have fimbriae, and as yet their significance is uncertain.

Capsules. 110, 111 Many bacteria, probably all if grown under appropriate environmental conditions, are surrounded by a layer of gelatinous, poorly defined, and poorly staining material that has been variously termed the capsule, slime layer, or envelope. While there is a tendency to apply one term to one kind of bacterium and another to another kind, viz., the capsule of the pneumococcus, the slime layer of Leuconostoc, and the envelope of the plague bacillus, the terms are, for all practical purposes, interchangeable. Among the pathogenic bacteria, the presence of a capsule is associated with virulence, not because of

any essential function of the capsule in the economy of the cell, but because it interferes effectively with the ingestion of the bacteria by the phagocytic cells of the body.

Bacterial capsules are not seen in the usual stained smear because of their failure to retain the dye. They may be faintly stained by some procedures, ²⁸ and alcian blue has been reported ⁶³ to stain the capsular polysaccharide, but in general the most satisfactory method of demonstrating the capsule is the "relief" preparation, wet or fixed and dried, in which the bacteria are suspended in diluted India ink (colloidal carbon). The capsule displaces the ink, and the bacterial cells appear to lie in lacunae, representing the capsules, in the dark background.

The relative size of the capsule varies widely. In heavily encapsulated forms, such as pneumococcus and Friedländer's bacillus, the thickness of the capsule is frequently greater than the diameter or breadth of the cell and is often continuous over adjacent cells such as diplococci or streptococci. In other bacteria it may be much smaller, no more than a thin layer about the cell, but still demonstrable, and in still others the capsule is too small to be directly demonstrable even though capsular material may be shown to be present by chemical and immunological methods. The size of the capsule is somewhat dependent upon the method used for its demonstration, i.e., drying and fixing tends to shrink it; it is generally agreed that wet relief preparations give the most accurate results.

The nature of capsular substance. The nature of the material making up the bacterial capsule has been of considerable interest, in large part because in a number of instances the formation of antibody to the capsular substance of pathogens is the most significant element of the effective humoral immune response.

The capsular substance of most, but not all, bacteria is polysaccharide which may or may not contain nitrogen, and which may give sugar acids, amino sugars, and organic acids as well as monosaccharides on hydrolysis. For example, the capsular material of type 2 pneumococcus is a polymer of glucose. That of type 3 pneumococcus is more complex and consists of glucose and glucuronic acid linked at the 1 and 4 positions respectively to give $4-\beta$ -glucuronosidoglucose, or cellobiuronic acid, and these

aldobionic acid units are polymerized by union at positions 1 and 3 to give chains having molecular weights as high as 140,000. The capsular substance of type 1 pneumococcus contains nitrogen and appears to be a polymer of trisaccharide made up of two uronic acid (in part galacturonic acid) moieties together with a third component containing nitrogen and acetyl groups.

Some bacterial capsules may contain mucoprotein admixed with polysaccharide, but whether this is to be regarded as a constituent of the capsule or a contaminant is not clear. However this may be, the marked superiority of the encapsulated bacterial cell to purified polysaccharide in stimulating antibody formation suggests that at least some of the polysaccharide may be linked to protein in the living cell.

Other bacterial capsular materials, especially in the genus Bacillus, are polypeptide in nature. That of the anthrax bacillus is a D-glutamyl polypeptide, while those of nonpathogenic species contain both D-glutamic acid and the naturally occurring isomer also, to as much as 20 per cent. In these forms the polypeptide does not appear to be mixed with polysaccharide. The capsular material of the tubercle bacillus seems to be a special case in that it contains relatively large amounts of nucleic acids admixed with polysaccharide.

The capsule is a loose structure that does not interfere with permeability. Since the enzymatic depolymerization of the capsular substances, such as the pneumococcal polysaccharide and the hyaluronic acid capsule of some hemolytic streptococci, appears neither to damage the cell wall nor affect viability, the capsule is regarded as a structure distinct from the cell wall in many bacteria. In some cases, however, notably the glutamyl polypeptide of Bacillus, there is evidence that depolymerization results in some damage to the cell wall, and it may be inferred that the capsular substance is also a part of the cell wall structure.

In a more restricted sense the capsule hardly attains the dignity of a cell structure in that the capsular substance is an end product of metabolism that is continuously secreted, or excreted, into the surrounding environment. Thus its size is a function of the rate of its formation and secretion and of its continuous solution at the periphery. While in most cases it is presumably formed

intracellularly, in some instances it may be produced outside the cell, perhaps at the cell surface. For example, cell-free culture fluid of *Leuconostoc mesenteroides*, a bacterium associated with slime formation in the sugar refining processes, polymerizes sucrose to the dextran capsular substance of this bacterium.

The specific capsular reaction. As indicated above, the capsular substance is antigenic, functioning largely as a partial antigen or haptene. It reacts specifically with homologous antibody to produce a microprecipitation in the capsule, altering its refractive index so that it appears better defined in wet unstained preparations.

This phenomenon was described in 1896 by Rogers, working with the fungus Oidium, and by Neufeld in 1902 for the pneumococcus, as a swelling of the capsule, the Ouellung reaction. This reaction allows rapid serological identification and has been applied to several groups of streptococci, the meningococcus, the influenza bacillus, Friedländer's bacillus, some of the coliforms. and to Pasteurella and Bacillus species, as well as to the pneumococcus. There appears to be some question as to whether actual swelling occurs. Careful measurement has shown that in most cases an increase in size cannot be demonstrated, but in some instances, such as pneumococcus, it may occur. Since one pneumococcus combines with 4×10^6 antibody molecules which, when packed, have a volume of about $0.8 \mu^3$, but increases in volume from 2.8 to 9.8 μ^3 , swelling in addition to the volume occupied by the immune globulin has occurred. 48 Such swelling may be a consequence of several factors such as a secondary hydration and an increased viscosity that retards diffusion.

The reaction of capsular substance with immune serum globulin also occurs with other proteins under appropriate conditions. It appears to consist of a salt-like combination between the capsular substance and proteins such as casein, various albumins and globulins, and hemoglobin at a low pH on the acid side of the isoelectric point of the protein. This capsular reaction differs from that with homologous antibody in that it is nonspecific and pH dependent.

STAINING REACTIONS

As implied before, the intact bacterial

cell stains readily with basic dyes such as crystal violet, methylene blue, and basic fuchsin but relatively poorly with acidic dyes such as eosin. While irregular staining with differentiation of more deeply staining areas or granules is observed in old cells and is characteristic of a few kinds of bacteria, most bacteria stain uniformly without the differentiation of internal structure that is readily demonstrable in, for example, mammalian tissue cells.

This marked affinity for basic dyes is indicative of an acidic protoplasm containing unusually large amounts of nucleoprotein more or less uniformly distributed. This inference is substantiated by a high content of purine and pyrimidine nitrogen in the bacterial cell, and ribonucleic and deoxyribonucleic acids make up from 5 to as much as 30 per cent of the dry weight of the cell substance.

The staining reactions of the intact bacterial cell are remarkably uniform with two important exceptions. These are the reaction to the Gram staining procedure and the high resistance to both penetration of dye and decolorization characterizing the so-called acid-fast bacilli.

The Gram-stain. This differential staining procedure was devised by the histologist Christian Gram as a method of staining bacteria in tissues, and described in 1884. It is an arbitrary procedure consisting of four steps, viz., (1) primary staining with crystal violet, usually containing a mordant such as ammonium oxalate; (2) the application of dilute (1:15) Lugol's iodine solution; (3) decolorization, most commonly with 95 per cent ethanol; and (4) counterstaining with a dye of contrasting color, usually safranin. When bacteria are stained by this method. they are separated into two groups. The gram-positive bacteria are those which retain the primary stain and are deep violet in color, and the gram-negative bacteria are those which are decolorized and are lightly stained by the counterstain, pink in the case of safranin.

The gram-positive reaction is relatively rare. It occurs only among the bacteria, yeasts, and filamentous fungi. A very few biological structures are gram-positive; they are those which are thought to be autore-producing, and they include chromosomes of certain species, mitochondria, centrosomes, and centromeres.

The reaction of bacteria to the Gram stain is correlated with a number of other characteristics, and representative differences are shown in the accompanying table. Such differences are only relative and are not completely consistent. The gonococcus, for instance, though gram-negative, behaves in many respects, such as susceptibility to the antibacterial activity of penicillin, as if it were gram-positive.

There are also significant differences in the permeability of bacterial cells to amino acids, glutamic acid and lysine in particular, that are associated with the Gram reaction. Gram-positive bacteria are distinguished by their ability to concentrate such amino acids from the environment, while their synthesis occurs intracellularly in gramnegative bacteria,34 an observation that may be linked with the relative complexity of the nutritive requirements of the gram-positive forms. Differences between bacteria correlated with the Gram reaction reach an impressive total and suggest that reaction to the Gram stain is a reflection of fundamental differences between the two kinds of bacteria. Consequently, the mechanism of the Gram reaction has been of considerable interest.

Three kinds of theories have been proposed to account for the Gram stain reaction. A differential permeability to the alcohol-soluble dye-iodine complex was proposed by Benians; a difference in isoelectric point of the cell contents between the gram-positive and gram-negative bacteria was urged by Stearn and Stearn on the assumption that a lower iso-electric point in the gram-positive bacteria would

result in a more stable complex with the basic dye of the primary stain; and, finally, it has been postulated, largely by Churchman, that the site of the Gram reaction is in the outer layers, or cortex, of the cell, the internal material being gram-negative in both kinds of bacteria.

Each of these is supported to some degree by available evidence, but none to the exclusion of the others. In general, the Gram reaction appears to be a property of the entire cell and to be associated with some cell components. The most precise evidence in this connection is the presence of a magnesium ribonucleate - protein complex in the gram-positive bacteria which is not present in gram-negative bacteria. This material can be removed from gram-positive cells by solution in bile to leave a gram-negative "cytoskeleton" and redeposited to make the cells gram-positive again. Gram-positive bacteria may also be made gram-negative by treatment with ribonuclease, and it has been possible to replace the bacterial ribonucleate with yeast ribonucleate. It has not, however, been possible to make naturally occurring gram-negative bacteria gram-positive by treating with the ribonucleate complex obtained from gram-positive bacteria.

There is also evidence associating the Gram reaction with other cell components, lipid and polysaccharide. Nevertheless, the mechanisms operative in the Gram reaction are still poorly understood, and the basic differences between the two kinds of bacteria, associated with or reflected in other properties, are not resolved.

The acid-fast stain. It was early observed that some kinds of bacteria are difficult to

Differences Between Gram-Positive and Gram-Negative Bacteria

GRAM-POSITIVE BACTERIA		GRAM-NEGATIVE BACTERIA
More susceptible	Antibacterial activity of basic dyes, anionic and cationic detergents, phenol, sulfonamides, penicillin	More resistant
More resistant	Antibacterial activity of azides, tellurites, oxidizing agents, streptomycin	More susceptible
More resistant	Digestion by proteolytic enzymes, lytic action of alkali	More susceptible
More complex	Nutritive requirements	Simpler
11-22% lipid	Cell wall composition	1-4% lipid
All amino acids present		Lacking some amino acids

stain and equally resistant to decolorization with highly effective agents such as acid-alcohol, and because of the latter were termed acid-fast. In this respect they resemble superficially the bacterial spore. These bacteria form a homogeneous group, the acid-fast bacteria, making up the genus Mycobacterium, which includes the tubercle and leprosy bacilli together with pathogens of cold-blooded animals and saprophytic forms.

These bacteria are characterized by a high, as much as 40 per cent of the dry weight, lipid content. These bacterial lipids have been studied in some detail, especially those of the tubercle bacillus, and have been found to consist largely of phospholipid and wax. The unsaponifiable portion of the wax fraction includes higher alcohols, one of which, a saturated hydroxymethoxy acid named mycolic acid, is associated with the property of acid-fastness. Mycolic acid may be regarded as the substrate in this staining reaction, and the property of acid-fastness, confined to these bacteria and some actinomycetes, appears to be a consequence of a relatively greater solubility of the phenoldye in the cellular lipids than in the decolorizing agent.

Neither the Gram reaction nor the acidfast property of some bacteria gives information about the morphological structure of the bacterial cell, but they are concerned with its chemical structure. In the first instance chemical structure is associated with other properties of the cell as noted above, and in the second with immunological phenomena and pathogenesis in the case of pathogenic forms.

THE CELL WALL AND PLASMA MEMBRANE

The occurrence of bacteria in shapes other than spherical is evidence of a rigidity of structure sufficient to withstand the forces of surface tension. While the gel-like state of the protoplasm may contribute in part to such rigidity, the cell structure largely responsible is the cell wall. It was not possible to demonstrate unequivocally the presence of a cell wall in bacteria until relatively recently when newer techniques, especially electron microscopy, became available. It is now apparent that the outer structure of the bacterial cell is dual in nature and consists of the cell wall proper and the plasma membrane which lines the inner surface of the cell wall.

The cell wall. 80, 89, 103 The cell wall is not apparent in the usual stained smear, but when the cytoplasm has been shrunk away by boiling the cells in dilute alkali it is demonstrable in crystal violet-stained smears. It may also be stained by Dyar's method in which Congo red is used with cetyl pyridinium chloride as a cationic mordant.

When the bacterial cell is shattered by physical means such as sonic or ultrasonic vibrations, the cell wall fragments do not tend to round up but show jagged lines of fracture. In electron micrographs of cell wall fragments the structure appears to be a collapsed sac, but this is an artifact due to

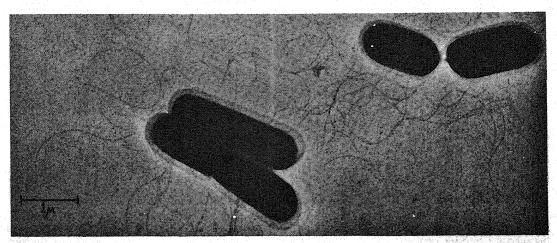


Figure 10. Electron micrograph of Clostridium tetani from 24-hour culture showing the clear cell walls and peritrichous flagella. (Mudd and Anderson.)

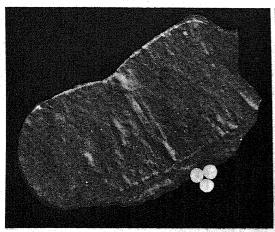
the effects of surface tension forces operative during the process of drying. When the cell walls are dried from the frozen state this is avoided, and rod-shaped forms, for example, are cylindrical in shape. Data such as these strongly support the concept of the cell wall as a structure having rigidity as well as ductility and elasticity apparent in the intact cell.

Cell wall preparations. Bacterial cell walls may be prepared free of intracellular material in a number of ways. Mechanical disintegration of the cells has the advantage that it avoids chemical alteration of the cell wall substance, provided that autolysis by the freed microbial enzymes does not occur. This is accomplished by grinding, shaking with small glass beads, or by sonic or ultrasonic disruption.

Bacteria vary in their resistance to such mechanical disruption. In the Mickle tissue disintegrator,68 in which the bacterial suspension is mixed with small glass beads such as glow beads and the containers shaken by a vibrating tuning fork, the time required for disruption of 95 per cent or more of the cells varies from 5 minutes for fragile bacteria such as the cholera vibrio to as much as an hour for staphylococcus. Similar results are obtained by subjecting the bacterial suspension to sonic vibration at 9 to 10 kc. or ultrasonic vibration at 20 to 40 kc. Some bacteria are highly resistant to the latter kind of treatment, especially staphylococci and streptococci, and in general gram-positive bacteria are more difficult to break up by mechanical means than are gram-negative bacteria. The cell walls are separated from the liberated cell contents by centrifugation and, after repeated washing with distilled water or molar sodium chloride solution, are free of electron-dense particles, nucleic acids, etc. Cell walls so prepared retain substantially all their original properties, for example, will adsorb bacteriophage (Chap. Four), are stable and resistant to digestion with trypsin, ribonuclease, and deoxyribonuclease.

Properties of the cell wall. Studies on such preparations have shed considerable light on the nature of the cell wall. It is thinner in gram-negative bacteria than in grampositive bacteria, ranging in general from 10 to 25 m μ in thickness, makes up about 20 per cent of the dry weight of the cell, has an effective pore diameter of 1 to 2 m μ , and is permeable to molecules are large as nucleotides.

While the cell walls of most kinds of bacteria appear to be homogeneous in electron micrographs, those of others, notably Spirillum, are made up of spherical macromolecules, ranging from 50 to 150 m μ in diameter but constant within species, arranged in regular, hexagonal or rectangular, patterns. 44, 53, 90 Assuming a regular packing of such macromolecules, the interstices correspond approximately to the effective pore diameter determined by permeability studies.



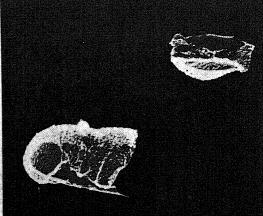


Figure 11. Cell walls of *Bacillus megaterium*. Left, an air-dried preparation which superficially exaggerates the size of the cell; the thickened band represents cell wall synthesized during the early stages of cell division, and the spherical particles are latex. × 11,500. Right, freeze-dried preparation showing the three dimensional structure of the cell wall, corresponding more closely to the size of the intact cell. × 12,250. (Salton and Williams.)

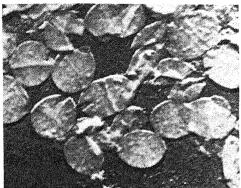




Figure 12. Cell wall preparations. Left, unwashed cell walls of Streptococcus fecalis prepared in the Mickle apparatus, showing the splitting of the cell wall which allows the contents to escape, the small electron-dense bodies adhering to the cell wall, and the thickened bands occurring in the early stages of cell division. \times 12,000. Right, freeze-dried preparation of the cell wall of Rhodospirillum rubrum showing the spherical bodies of the cell wall of this microorganism. \times 42,000. (Salton and Williams; Salton and Horne.)

The cell wall substance consists of peptide, polysaccharide, and lipid, highly individualistic in kind and amount in different bacterial species. 119 The hydrolysis products of the peptide include glucosamine, D- and L-alanine, D-glutamic acid and either Llysine, DD- or LL- or $meso-\alpha-\epsilon$ -diaminopimelic acid, 2,4-diaminobutyric acid, or either D- or L-ornithine. These substances occur in the cell wall as covalent-linked polymers, and have been termed mucopeptides, glycopeptides, glyco-amino peptides, or mureins, differing in composition from one kind of bacteria to another. The mucopeptide structure appears to be the mechanical support maintaining the shape of the bacterial cell.

The polysaccharides hydrolyze to sugars such as rhamnose, galactose, glucose, mannose, and arabinose. That of the diphtheria bacillus has been found,⁴¹ for example, to be an oligosaccharide made up of two molecules of D-galactose, one of D-mannose, and three of D-arabinose, and to have a molecular weight of about 1000. Hexosamine is found regularly and probably exists in the cell wall as N-acetylhexosamine though not as a chitin-type polymer.

Relatively large amounts of phosphorus are present as ribitolphosphate and glycerophosphate polymers. These complexes contain other substances, e.g., α -glucosyl and O-alanyl residues, and are called teichoic acids.

While, as indicated above, bacterial cell walls are not digested by trypsin, those of a number of kinds of bacteria, including cer-

tain of the micrococci and Bacillus, are broken down by lysozyme, the bacteriolytic agent found in lacrimal and other secretions, egg white, etc. The substrate of lysozyme in the bacterial cell wall is a hexosamine-containing mucocomplex or mucopolysaccharide, but the enzymatic breakdown is incomplete in that the major products of degradation have molecular weights of 10,000 to 20,000. The dissolution of the cell wall by lysozyme, then, is not a matter of complete digestion, but rather a degradation of a component of the cell wall that may bind the major peptide and polysaccharide components, with breakdown of the cell wall as a structure. Lysozyme hydrolyzes the β 1-4 linkages between N-acetylmuramic acid and N-acetylglucosamine, and the cell walls of lysozyme-sensitive bacteria contain appropriately linked repeating units of these substances.

It has been observed also that the cell wall is depolymerized as a supporting structure in immune lysis (Chap. Fourteen); this has been attributed to the activity of lysozyme present in the serum reagents. In at least one instance,³¹ however, cell wall is depolymerized in the presence of the antibodycomplement complex in the absence of demonstrable lysozyme, and the osmotic fragility of the resulting protoplasts (see below) produces death of the cells in the immune bactericidal reaction.

The cell walls of streptococci are resistant to the action of lysozyme but are broken down by enzymes produced by *Streptomyces albus* and other actinomycetes.^{62, 88}

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Bacterial cell walls attacked by lysozyme are not affected by these enzymes, indicating that a different substrate is involved. The products of degradation are, however, similar to those produced by lysozyme.

The plasma membrane. The plasma, or cytoplasmic, membrane of the bacterial cell lies on the inner surface of the cell wall, separating it from the cell plasma. On mechanical disruption of the cell, fragments of the membrane may be seen attached to the broken cell wall, and it may be stained in the cell by appropriate techniques. The membrane, on separation from the cell wall and debris of fragmented cells, breaks up into the so-called small particle fraction that may be removed by differential centrifugation.

The plasma membrane substance appears to be a complex lipoprotein, containing 40 per cent protein and 20 to 25 per cent lipid, and makes up about 10 per cent of the dry weight of the cell.⁷¹ These and related data may be taken to indicate that it conforms to the classic concept of lipoprotein membranes and is, in the intact cell, a closely packed lipoprotein sheet, two to four molecules thick, and about 15 m μ thick in the hydrated form.

The bacterial membranes differ from mammalian membranes in a number of ways: viz., sterols appear to be absent except in the case of Mycoplasma, little or no phospholipid of the glyceryl phorphoryl choline type is found though glyceryl phorphoryl ethanolamine lipids are present, there are relatively large amounts of unesterified polyglyceryl and glyceryl phosphates, and oddnumbered branched chain fatty acids are present in large proportion. The significance of such differences in chemical composition is as yet uncertain.

This membrane has little mechanical strength and does not contribute significantly to maintenance of cell shape as shown by the rounding up of the bacillary form after removal of the cell wall by enzymatic digestion (see below). It is forced against the cell wall by a hydrostatic pressure, as much as 20 atmospheres, corresponding to the osmotic pressure differential across it. While it is a structure distinct from the cell wall in that the two differ in chemical composition and enzyme content, the two are joined by more than lateral cohesion. Thus, the plasma membrane is broken up in the

absence of the cell wall by chilling, but much less readily in the intact cell, and it may be inferred that some sort of bonding occurs between the two structures such that rearrangement of parts of the membrane to give freely permeable areas is inhibited.

Osmotic regulation.⁷² While the cell wall and plasma membrane are distinct, artificially separable structures, in the bacterial cell they make up an integrated unit whose function is, in part, that of an osmotic barrier and regulatory mechanism separating the cell plasma from the suspending medium and implementing, for the bacteria, Claude Bernard's concept of the relatively isolated environment required by free-living organisms.

The contribution of the cell wall to this joint function is largely that of a supporting structure, but it also contributes to the permeability of the intact cell in that it acts as a barrier to substances having molecular weights of 10,000 or more. While this is not essential to the physiological economy of the cell (see below), it does provide some protection against large molecular weight substances such as antibodies and lytic enzymes. The plasma membrane functions as the significant osmotic barrier to lower molecular weight substances and exhibits the usual properties of living semipermeable membranes such as selective permeability and the transport of substances across the barrier against concentration gradients.

The bacterial cell is apparently freely permeable to the sodium ion, with rapid equilibration between internal and external concentrations. The potassium ion, however, may accumulate intracellularly against a concentration gradient; the process is associated with exergonic metabolism and is inhibited in the presence of 2-4-dinitrophenol, sodium azide, and similar respiratory poisons.

Relatively precise studies of bacterial permeability to amino acids, lysine and glutamic acid particularly, have been carried out with a few species of bacteria, and there appear to be significant differences between the gram-negative and gram-positive forms. It is generally said that the gramnegative bacteria are freely permeable to amino acids while the gram-positive bacteria are not. This appears not to be strictly true for, on the one hand, the occurrence of internal amino acid pools is demonstrable

in at least some gram-negative bacteria, and, on the other, the gram-positive bacteria studied seem to be at least partially permeable to some amino acids although the accumulation of others involves active transport and a source of energy. Thus, while lysine appears to diffuse readily in both directions across the osmotic barrier, and is independent of exergonic metabolic activity, at the same time intracellular lysine cannot be washed out of bacterial cells suspended in distilled water unless glucose is present.

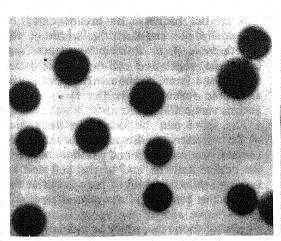
Permeases.25 There is evidence that the active transport of nutrient substances across the osmotic barrier is a function, at least in part, of enzymatic activities to which the name permeases has been given. Such a permease is essential to the utilization of lactose by some strains of coliform bacilli. e.g., the appropriate enzyme, β -galactosidase, may be present within the cell, but unless the transport mechanism is present, the sugar is not utilized. The permease activity is genetically controlled, and cells which lack it, but contain β -galactosidase, are designated cryptic mutants (Chap. Seven), i.e., metabolically inert in the presence of the substrate. A considerable number of such permease activities have been described.

Bacterial permeability is of particular interest in connection with the nature of the effect of antibacterial substances characterized by bacteriostatic or growth-inhibiting activity such as the chemotherapeutic drugs, the basis of reversal of antibacterial

activity, and the nature of drug resistance as related, for example, to altered permeability to amino acids such as glutamic acid.³³ The evidence, though meager in contrast with that available for classic subjects of permeability studies such as Arbacia eggs and erythrocytes, suggests that an active transport mechanism or exchange diffusion is involved, with reversible deformation of parts of the plasma membrane to allow passage of the carrier.⁶⁹ This raises the interesting possibility that some kinds of antibacterial activity may result from effects on a carrier mechanism and/or the structure of the osmotic barrier.

Bacterial protoplasts. 13, 65, 115 On enzymatic degradation of the cell wall of the living bacterial cell, as for example by treatment of Bacillus species with lysozyme, the cell first swells, then the protoplasm becomes partially detached from the disintegrating cell wall, and finally, following complete dissolution of the cell wall, the cell content assumes a spherical shape. This spherical form, the cell protoplasm enclosed in the plasma membrane, is the protoplast.

The protoplast is a fragile structure because the plasma membrane has little mechanical strength. When the cell wall is removed by enzymatic dissolution in physiological saline, the protoplasts begin to break up almost as soon as they are formed, first to an empty membrane or "ghost," followed by fragmentation of the membrane. 112 It was found by Weibull that sucrose and polyethylene glycol act as stabilizing agents, and it is now established that if



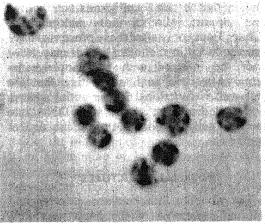


Figure 13. Protoplasts (left) and protoplast ghosts (right) of Bacillus megaterium. Note the spherical shape assumed by the bacillary form on dissolution of the cell wall. Phase contrast photomicrographs. × 3000. (Weibull.¹¹⁴)

the cell wall is removed from cells suspended in mediums such as 0.1 to 0.5 molar sucrose, 7.5 per cent polyethylene glycol, 0.25 to 0.5 molar sodium chloride, or 0.5 molar phosphate at pH 7, the protoplast is relatively stable over a period of several hours if kept under semi-anaerobic conditions and protected from shock. Stabilized protoplasts suspended in 0.2 molar sucrose solution containing magnesium salts at concentrations of 0.01 molar or greater may be lysed by reduction of the sucrose concentration to give stable "ghosts." When the cell wall is damaged so as to affect its structural integrity, but remains present, the bacterial cell also rounds up in spherical form, and is known as a spheroplast.

Physiological studies on stabilized protoplasts have shown that the competence of the intact cell persists almost, but not quite, entirely in the protoplast and indicates the function of the cell wall by implication. Protoplasts show a high endogenous respiratory rate $(QO_2 = 66)$ and can oxidize glucose. They are able to incorporate C14 in protein and nucleic acid at rates only slightly less than those of actively growing intact cells. The path of incorporation of acetate carbon is consistent with the operation of the tricarboxylic acid cycle in providing intermediates for amino acid synthesis⁶⁴ indicating that actual synthesis rather than exchange is involved, and the synthetic abilities of the protoplast are further substantiated by the synthesis of the adaptive enzyme β -galactosidase. There is some evidence also that the protoplast is capable of growth and division.

The protoplast differs from the intact cell in that it can form spores (see below) and support bacterial virus multiplication (Chap. Four) only after these processes have been induced in the intact cell. It may, however, be infected with bacteriophage infectious nucleic acid, suggesting that the cell wall is functional in the initial adsorption and discharge of the viral DNA into the host cell, but does not affect subsequent synthesis.

INTERNAL STRUCTURES 57, 76

The dividing line between external and internal structures of the bacterial cell is not a sharp one and is perhaps justified for the most part on little more than a utilitarian

basis. Thus, in a sense the protoplast is a subcellular element, yet flagella remain attached to it since the basal granules at or near the inner surface of the cell wall in which they originate are apparently not an integral part of the cell wall structure because they remain after enzymatic dissolution of the cell wall. Similarly, the bacterial spore is formed within the cell, but it represents a stage in the life history of the bacterium rather than a subcellular structure in the ordinarily accepted sense.

spore. 30, 36, 104, 108 The bacterial formation is uncommon among the bacteria and is confined largely, if not entirely, to the bacilli. The aerobic spore-forming bacilli make up the genus Bacillus, and the obligate anaerobic or micro-aerophilic bacilli that form spores are members of the genus Clostridium. Both are widely distributed in nature. Members of the former group are saprophytic forms found in soil, dust, and water and, with the exception of the anthrax bacillus, are not pathogenic for man under ordinary circumstances. The anaerobic group is similarly distributed and includes both saprophytic nonpathogenic forms together with microorganisms which are pathogenic by virtue of toxin formation, such as the tetanus and gaseous gangrene bacilli and the botulinus bacilli, although they are not obligate parasites.

The bacterial spore is a refractile oval body formed within the bacterial cell and found both intracellularly and extracellularly in the usual stained smear; the vegetative cell substance has disintegrated about those lying free. Within the cell, the long dimension of the spore is parallel with the long axis of the bacillus. Its breadth may be essentially the same as the width of the vegetative cell, or it may be greater and bulge the vegetative cell wall. The latter appearance is most commonly found in the anaerobic forms and is the basis of the generic name Clostridium.

The spore may be located in the center of the vegetative cell, where it is said to be central, or it may be found part way between the center and the end of the cell and be subterminal, and finally it may occur at the end of the cell and is designated terminal. The relative size of the spore is constant, and its location reasonably so within species. Its size and location may result in a characteristic morphology, e.g., the large ter-

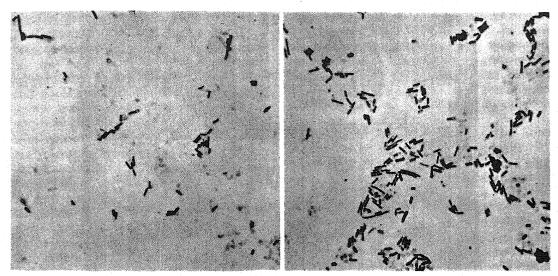


Figure 14. Spore formation by pacteria. Left, Clostridium botulinum type B, showing the typical clostrispores separated from disintegrated vegetative cells. Right, Clostridium sporogenes, showing a subterminal clostridial spore. These preparations are all stained with a single stain in the usual way; the vegetative cells take up the dye, but the spores remain unstained. Fuchsin; × 1050.

minal spore of the tetanus bacillus gives the spore-containing vegetative cell a drumstick appearance.

In the usual stained smear the spore appears as an unstained refractile body within the stained vegetative cell, and some stain may adhere superficially to free spores to accentuate their outlines. In electron micrographs of ultrathin sections, it is apparent that the spore consists of a dense central core, a fibrillar cortex, and an outer coat. The spore may be stained by using heat and/or mordants. Once stained, it is equally difficult to decolorize with the usual reagents such as alcohol, acid-alcohol, and alcoholacetone. Consequently, differential staining of the spore is a simple matter; for example, the smear may be stained with hot carbolfuchsin, decolorized with alcohol, and counterstained with a dye of contrasting color, such as methylene blue to give a red spore in a blue vegetative cell.

The spore has a low, but measurable, endogenous metabolism, about $0.3~\mu l.~O_2$ consumed per hour per mg. dry weight. It is characterized by an increased calcium content over that of the vegetative cell, and the presence of dipicolinic acid (pyridine 2,6-dicarboxylic acid) contained in the outer coat.

The factors involved in spore formation are poorly understood, but in general spores are formed most readily under optimal con-

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ditions for growth, and spore formation begins toward the end of, or just following, the period of exponential growth. There is no reason to believe, as is sometimes supposed, that spore formation occurs in response to adverse environmental conditions.

The effects of environmental conditions on spore formation vary from one kind of bacteria to another. For example, the aerobic anthrax bacillus forms spores only under aerobic conditions, and spores are not found in impression smears of organs such as spleen from animals dead of anthrax since the tissues are not sufficiently aerobic to permit sporulation. Similarly, the anaerobic sporulating bacilli do not form spores under aerobic conditions that do not allow growth. The temperature of incubation may be a factor also, and it is common knowledge that aerobic sporulating bacilli do not form spores when grown at maximal temperatures tolerated for growth, and, in fact, permanently asporogenous variants may be produced by continued cultivation at high temperatures.

Cytological observations have shown that at the initiation of spore formation there is an assembling or local concentration of material staining deeply with basic dyes, presumably nucleoprotein, which constitutes the spore primordium or forespore. This may occur with the formation of a granule which enlarges to form the spore in some bacteria, in others there seems to

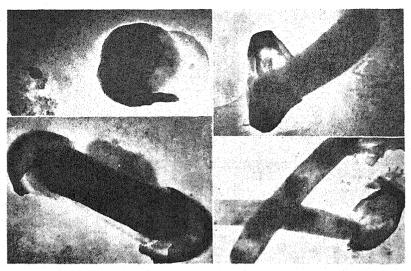


Figure 15. Electron micrographs illustrating the process of germination of spores of *Bacillus mycoides*. In the *upper left* the vegetative cell is just beginning to emerge from the spore case. In the *upper right* the vegetative cell is growing out of the side of the spore case and has broken the case in two parts in the *lower left*. In the *lower right* germination is complete. The darker areas in the vegetative cells are regarded by some as nucleus-like in nature. (Courtesy of Dr. G. Knaysi and the Journal of Bacteriology.)

be an aggregation of deeply staining granules, and in still others only a local concentration unrelated to granules. In any case, when formation is complete the spore assumes the characteristic refractile appearance, and the surrounding vegetative cell sloughs off. Only one spore is formed by a single vegetative cell, and spore formation cannot be regarded as a method of multiplication, and, further, there is no evidence of sexual phenomena or conjugation in the formation of bacterial spores such as occurs among the fungi.

It has been found²⁷ that the spore contains antigens that are not present in the vegetative cell, and this indicates that the process of sporulation is more than an assembling and concentration of vegetative cell substance. The spore antigens are specific in that spores from immunologically different vegetative cell species are distinct and do not share common spore antigens. The spore contains a full complement of the enzymes present in the vegetative cell, though in reduced amount, and is not dormant in that the low endogenous respiration of moist spores is measurable in the usual respirometers.

The composition of the spore does not account satisfactorily for its unusual resistance to injury although the presence of dipicolinic acid is considered by some to be associated with heat resistance. It is not only relatively impermeable to dyes, as

noted above, but also is much more resistant to the injurious effects of heat, drying, many bactericidal chemical compounds, etc., than is the vegetative cell.

Sterilization procedures (Chap. Six) are necessarily directed toward the destruction of the ubiquitous microbial spore. Some bacterial spores, such as those of organisms causing flat sours in canned foods, may be extremely resistant to heat and require heating in steam under pressure at 120° C. for as long as three hours to insure destruction. Most are not this resistant and are killed by moist heat at 115° to 120° C. for 15 to 20 minutes. Still others are destroyed by boiling for a short time, but few if any are killed by treatment at 58° to 60° C. for 30 minutes, which suffices to destroy most bacterial vegetative cells. They are also extremely resistant to drying; the classic example is that of the viability of anthrax spores dried on silk threads in Koch's laboratory after 60 years.

The ratio of the concentration of bactericidal compounds lethal to the vegetative cell to that lethal for the spore is on the order of 10³ to 10⁴ for many disinfectants such as hypochlorites and phenols. Interesting and significant exceptions are the alkylating agents such as ethylene oxide or formaldehyde, for which the ratio ranges from 0.5 to 15.

When the spore is placed in an environment favorable to the growth of vegetative cells it germinates. The factors affecting germination are not well known. It may be delayed and irregular, and in some instances this has been found to be associated with the presence of fatty acids, such as oleic and linoleic acids, in the culture medium. Spores may be germinated mechanically by abrasion of the spore coat with glass heads. with consequent loss of dipicolinic acid.86 but release of dipicolinic acid with surface active agents does not allow further development.87 Or germination may be accelerated by heat shock, a short exposure to a high temperature, say 80° C., prior to culture. Once germination is initiated, it occurs at an exponential rate. Nucleic acid metabolism differs from that of the vegetative cell. at least in the case of Bacillus subtilis, with a preliminary synthesis of RNA during the first two hours, followed by initiation of DNA synthesis. Dipicolinic acid is excreted during germination and is not found in germinated spores or vegetative cells.

The first microscopic evidence of germination is a change in the refractile appearance of the spore, and it becomes more permeable to dyes and takes up dye like the vegetative cell. The sequence of events thereafter is variable. The spore wall may become thin and stretch and assume the shape of the vegetative cell. In other instances the vegetative cell appears to grow within the spore wall which is split and cast off as a hull. The anthrax bacillus grows out of one side of the spore wall, while the



Figure 16. Recently germinated vegetative cells of Bacillus mesentericus, showing discarded spore cases. Note the cell boundaries in the long filaments made up of cells attached end to end. The deeply staining areas are chromatinic bodies. Osmium tetraoxide-hydrochloric acid fixation, Giemsa; × 4000. (Robinow.)

closely related *Bacillus subtilis* grows out of opposite sides simultaneously. In *Bacillus megaterium* the spore case splits along the equatorial ridge or groove, allowing the vegetative cell to emerge. Other bacteria have intermediate methods of germination, and irregularities may occur in the development of spores into vegetative cells in the same bacterial species. It is to be noted that, as the vegetative cell produces only one spore, on germination the spore gives rise to only a single vegetative cell, and the process of germination is not of multiplication.

The biological function of the bacterial spore is not known. It is clearly a normal stage in the life history of the spore-forming bacteria, and may be regarded as a resting stage, possibly analogous to hibernation or estivation in higher forms. Its high resistance to injury serves to carry the bacterial species through unfavorable circumstances, and the spore might be supposed to have survival value.

The bacterial nucleus. 32 The bacteria contain very large amounts of nucleic acids by analysis, more than most other kinds of cells, and the usual uniform staining of the bacterial cell by basic dves is indicative of their general distribution within the cell. Like other organisms, but with the exception of the viruses (see below), bacteria contain both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The DNA is contained largely in masses or bodies within the cell, while the RNA occurs in the surrounding cytoplasm. This localization of DNA is masked in the conventional stained preparation since the RNA is stained also, but is demonstrable by staining if the RNA is selectively removed by mild acid hydrolysis or treatment with ribonuclease, or is deficient as in starved cells growing on a nutritionally minimal medium. The masses of DNA, or chromatinic bodies, generally stain only weakly with the basic dyes, though they may be stained with mordant and fuchsin, but stain strongly with Giemsa and are Feulgen-positive.*

^{*}The Feulgen reaction is a microchemical test for the presence of DNA. The aldehyde group of acidhydrolyzed DNA react with Schiff's reagent to restore the color to the reduced fuchsin, and aggregates of DNA appear as violet-colored masses. The test is one for free aldehyde groups and is not specific for DNA.

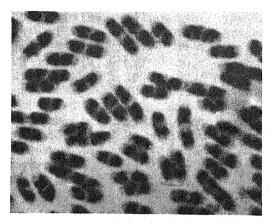


Figure 17. Light microscope photomicrograph of a two and one-half hour culture of colon bacilli, fixed with osmium tetraoxide vapor and alcoholic mercuric chloride, hydrolyzed at 56° to 60° C. with normal hydrochloric acid, and stained with Giemsa. The deeply staining bodies are chromatinic bodies. (Robinow.)

It is obvious from the persisting identity of a given strain of bacteria that a heredity-controlling mechanism must be present, and it has become clear that the chromatinic bodies are nuclei with respect to function. There are one to two per cell, or three to four in actively multiplying bacteria, and they replicate coincident with cell division to occur in the daughter cells. At the same time, they are relatively simple in structure and are not enclosed within a limiting membrane, and reports of the occurrence of mitotic figures seem not to be well founded. There is, therefore, some reservation as to the complete identity of the chromatinic

bodies with nuclei in the conventional sense, and they are commonly termed nucleoids.

The morphology of the bacterial genetic apparatus has been determined in large part from studies on the recombination of inheritable characters during bacterial coniugation38 (Chap. Seven). It has been established that individual genetic determinants are arranged in linear order, and the entire genome is contained in a single DNA duplex which, when fully extended, is 100 to 1400 μ long. This structure has been directly observed in elegant experiments by Cairns¹⁹ and by Kleinschmidt and co-workers. 51 In the first instance tritium-labeled DNA was released by lysis of the bacterial cell with Duponol and allowed to disentangle on the surface of a dialysis membrane, which was in turn overlaid with photographic emulsion. and radioautographs were made. In the second instance protoplasts were lysed in a spreading monomolecular protein film at an air-water interface, shadow-cast, and examined by electron microscopy. No free ends were observed, and this is consistent with the inference from conjugation studies that the E. coli chromosome is circular in contrast to that of T₂ coliphage, which is linear. But transfer during conjugation is linear, and it is evident that the circular chromosome must be broken to allow such transfer. The mechanism by which breakage occurs and polarization for transfer takes place is highly controversial. Evidence has been adduced, largely by Jacob, Monod, and their co-workers, that the point

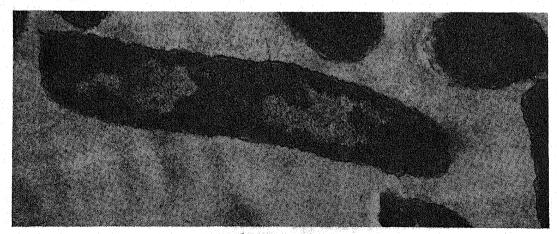


Figure 18. The DNA chromatinic bodies in *Escherichia coli* K-12S during the exponential growth phase. Note the apparent lack of a limiting membrane. Section fixed in osmium tetraoxide. × 40,000. (Kellenberger.)

at which the circular chromosome is opened lies within the sex factor (Chap. Seven) and the chromosome is in contact with the plasma membrane at this point, but this is not unequivocally established.

Since the bacterial chromosome is perhaps a thousand-fold longer than the nucleoid, it follows that it must be folded or otherwise compacted to be contained within the nucleoid. Electron microscopy of ultrathin sections has made it clear that the most general characteristic feature of the structure of the nucleoid is a parallel arrangement of DNA aggregates containing perhaps 500 molecular helices of DNA and preferably oriented to the long axis of the bacterial cell as described by Kellenberger. 50 It seems probable that these aggregates represent DNA fibers folded back and forth. If so, then the question of the postulated contact of the chromosome with the cell membrane is raised. Such contact is demonstrable in electron micrographs, and it is found that in the protoplast there is such a direct contact to the plasma membrane, and in the bacterial cell there is contact, or even penetration, between the nucleoid and invaginations of the plasma membrane (known as mesosomes, plasmalemmosomes, membranous organelles, etc.). It will be recalled in this connection that the nucleoid is not confined within a limiting membrane.

Cytoplasm. 46 The cytoplasm of bacteria appears to have a much less complex structure than that of other cells. The endocytoplasmic reticulum of the usual cell is lacking, particularly in gram-negative bacteria, and the membrane profiles seen in certain gram-positive bacteria are not clearly differentiated. There does appear, however, to be a network of presumably linear arrays with interconnections, and fine fibrils extend from the nuclear plasm deep into the cytoplasm.

Cytoplasmic granules.¹¹ The bacterial cell contains a variety of granules sufficiently large to be seen with the light microscope. Those staining with certain basic dyes are known as metachromatic or Babes-Ernst granules, and are readily observed in bacteria such as the diphtheria and related bacilli stained with alkaline methylene blue or toluidine blue and as polar granules in other forms such as plague bacilli fixed in methanol prior to staining. They are variable in size, to as large as 0.6μ , and larger

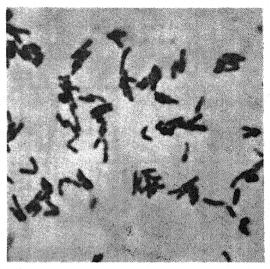


Figure 19. Metrachromatic granules and bipolar staining in the diphtheria bacillus. Note the differences between these organisms and the plague bacilli. Methylene blue: × 1975.

granules may represent aggregates of smaller ones. These granules contain relatively large amounts of trichloracetic acid-insoluble polymetaphosphate whose presence is associated with their metachromatic character. They are dissolved in dilute acid or alkali or boiling water and are characteristically electron-dense but are destroyed in an intense electron beam.

Other visible granules are polysaccharide or lipid in nature. The former stain with iodine to a red-brown color, and the material composing them has been variously termed iogen, granulose, or bacterial starch. Intracellular polysaccharide may also be stained by the periodic acid-Schiff method. Lipid granules have the appearance of vacuoles in bacteria stained with basic dyes but may be stained with lipid-staining dyes such as Sudan III in wet preparations or Sudan Black in dried smears.

The granular polysaccharide and lipid may be regarded as end products of metabolism, and they appear to play no active part in the physiological processes of the cell. Metachromatic granules, however, are the site of enzymatic activity (see below), and the contained polyphosphate serves to store energy conserved in high energy phosphate bonds, accumulated during oxidative processes with adenosine triphosphate (ATP) acting as the intermediate. The term "reserve substance," which has long been

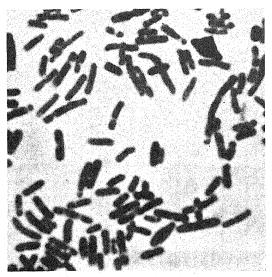


Figure 20. Irregular and bipolar staining of the plague bacillus. Note the heavily stained areas and metrachromatic granules. Fixed in methyl alcohol and stained with methylene blue; × 2400.

applied to metachromatic granules without supporting evidence, acquires significance in this way.

Submicroscopic particulates. In addition to the granules that may be seen with the light microscope, bacterial protoplasm, like that of other cells, contains minute granules or particulates that are separable from soluble constituents of the cell by centrifugation at speeds giving $100,000 \times G$ or more and may be observed only in electron micrographs. It is somewhat disconcerting that such particles are not seen in electron micrographs of ultrathin sections, and it is questionable whether they occur in the bacterial cell in the same form as that found in lysates.

The sedimentation patterns obtained with bacteria disrupted by various means, and exclusive of large fragments such as cell wall, metachromatic granules, and chromatophores, consistently show several distinct macromolecular components. There is usually a broad, slowly moving band at 5 S,* and peaks at 8 S, 20 to 30 S, and 40 S.

The 5 S fraction is a heterogeneous mixture of substances making up the ground material of the protoplasm. The 8 S fraction contains the major portion, 80 to 90 per cent, of the DNA present in the cell. The latter suggests that morphological organ-

*Svedberg units, i.e., S = 10a¹³ cm./sec./unit field.

ization of DNA in the intact cells, as for example in chromatinic bodies or other structures that might be thought to have nuclear organization, is fragile inasmuch as constituent DNA is readily liberated in soluble form. This fraction also contains the unstructured RNA present in the cytoplasm. Together these two fractions constitute the supernatant and contain half or more of the total intracellular substance measured as nitrogen, protein, or dry weight.

RIBOSOMES.66 Of the submicroscopic particulates, the ribosomes have been of special interest since they are intimately concerned with protein synthesis. The cytoplasmic RNA present in the bacterial cell is separable into three kinds on the basis of function, viz., ribosomal RNA (r-RNA), amino acid transfer, or transfer, RNA (t-RNA), and messenger RNA (m-RNA), and these are also differentiable with respect to composition and molecular weight. Further subdivision may be made which does not concern us here. The nucleoid contains DNA's that are complementary in nucleotide sequence to all three kinds of RNA, and these RNA's are synthesized on these templates, r-RNA and t-RNA continuously, and m-RNA at a variable rate depending upon the presence of repressors or inducers with a maximum rate of about one copy site in two to three seconds.

The r-RNA is complexed with protein and presumed to occur in the cell as the 70 S fraction, though particulates of this size have not yet been observed in electron micrographs of ultrathin sections. There appear to be about 12,000 such ribosomes per cell the size of coliform bacilli. The protein moiety has a molecular weight of about 25,000 and constitutes about one-third of the complex. The 70 S complex is separable into two fractions having sedimentation coefficients of 30 S and 50 S, but the significance of this is uncertain.

The function of ribosomes in protein synthesis is dependent upon the function of the two other kinds of RNA which are, for the most part, soluble and present in structureless form in the cytoplasm. There are many kinds of t-RNA in that there are one to four kinds for each of the 20 amino acids. The amino acids are activated by a specific enzyme, and the residue transferred to a specific t-RNA. The m-RNA functions as the template for protein synthesis,

i.e., determines the order of amino acids in the peptide chain, and there are many different kinds of m-RNA, each corresponding to a specific protein. Operatively the appropriate t-RNA-amino acid complexes associate with the predetermined sites on the m-RNA template on the ribosome, each amino acid residue being brought into place by its t-RNA and displacing the previous t-RNA as the peptide chain grows.

Ribosomes occur, presumably in the cell, as aggregates of the 70 S particle (polysomes, polyribosomes) in variable number and linked by strands of m-RNA. This has been taken to indicate that a 70 S ribosome becomes attached to one end of an m-RNA. and progresses along its length as successive reactions with t-RNA's put into place in the peptide chain the amino acids dictated by the m-RNA template and the peptide chain is synthesized. Additional ribosomes may become attached to the same m-RNA, to give continuous synthesis of peptide in various stages of completion at the points of ribosome attachment. On reaching the end of the m-RNA, the completed polypeptide and ribosome are released, and the ribosome is again available to any m-RNA.

INTRACELLULAR LOCALIZATION OF ENZYMES^{60, 70}

It can be anticipated that the morphological differentiation of the bacterial cell into subcellular structures, both at microscopic and submicroscopic levels, may be associated with a corresponding physiological differentiation and biochemical organization. In fact the simultaneous occurrence of opposing processes, such as the synthesis, utilization, and destruction of nucleotides, within the cell makes some effective separation of metabolic processes inevitable.

The external cell structures, flagella and capsules, have no biochemical function, and their loss does not affect the physiological processes of the cell. The flagella serve only as macromolecular muscles so far as is known, and the capsular substance is a product of metabolism shed into the immediate environment, functional only insofar as it provides protection to the cell from, for example, phagocytosis.

Similarly, the cell wall seems to be largely a supporting structure that contributes in a

minor way to the osmotic barrier, but which also may be dispensed with in that the protoplast is physiologically complete with certain exceptions such as its inability to initiate sporulation. Cell walls washed free of protoplasmic contaminants, as judged by electron micrographs, have not been shown to have enzymatic activity.

The plasma membrane, as the most important element in the osmotic barrier of the bacterial cell, is closely associated with enzymatic activity. The intact membrane gives a positive Feulgen reaction as noted above, and the cytochrome system occurs in close proximity to it.

As noted elsewhere, the relative impermeability of the living bacterial cell to substances such as phosphate, coupled with the vigorous metabolic activity of these microorganisms, implies a highly effective transport mechanism across the osmotic barrier. If, as has been suggested, such a mechanism involved the adsorption of nutrient substances and desorption of end products of metabolism by the appropriate enzymes functioning as carriers, the association of enzymatic activity with the membrane would be very close even though the membrane itself may not have activity. Thus, it is plausible to assume that reduction of the cytochrome-cytochrome oxidase system occurs, at least in part at the periphery of the cell organization.55 Further, the facility with which electron-dense particles are trapped in fragments of cell walls of disrupted bacteria suggests that some of these particulates may lie in close proximity to the periphery of the cell.

Further evidence for the localization of enzymatic activity at the cell periphery is provided by, for example, the diffusion of glutamic acid into the cell and the association of glycolytic mechanisms with the cell surface. Glutamine penetrates the cell rapidly while glutamic acid does not unless a source of energy such as glucose is available, implying that the mechanisms of assimilation against a concentration gradient may involve decarboxylation at the cell surface. Similarly, the phosphorylation of hexose by yeast cells appears to occur largely at the periphery of the cell, and the enzymes catalyzing the dephosphorylation of adenosine diphosphate and inorganic pyrophosphate are localized there.

There appears to be little doubt that the

microscopic polymetaphosphate-containing metachromatic granules serve as a source of energy in high-energy phosphate bonds, but the way in which the energy available in these discrete "power houses" is tapped for endothermic metabolic reactions is unknown other than that it is released by way of ATP.

Treatment with tetrazoles, such as triphenyltetrazolium chloride, results in reduction to insoluble formazan which is seen to accumulate in localized areas. Similarly, indophenol blue appears in localized areas following treatment of the cell with the Nadi reagents, α -naphthol and dimethyl-pphenylenediamine. It has been objected that formazan accumulates by accretion in any case, and that indophenol blue may accumulate by solution in lipid granules, so that the granular loci of the indicated activities is an artifact. This criticism is not applicable to the observed sequential color changes in Janus green B which are typical of the mitochondrion and occur in intracellular bacterial granules or, though more slowly, in free granules present in bacteriophage lysates. While, then, these bacterial granules may not be mitochondria, there is evidence that they are the sites of enzymatic activity.

The greater part of the enzymatic activity of the bacterial cell is associated with the submicroscopic granules, and with the soluble substances, i.e., <20 S, present in the ground plasm. The particle size range is broad, although the bulk of the material occurs in the 40 S (10 to 20 m μ) fraction, but there appears to be no definite relation between size and localization of kinds of enzymatic activity. It is possible that the larger particles are aggregates, and the smaller particles fragments, of the 40 S fraction, i.e., the activity of even microscopic granules may be in part a consequence of the presence of occluded submicroscopic particles, while sedimentable enzymatic activity may be solubilized by treatment of sediments with sonic oscillation. Within these implied reservations, kinds of enzymatic activity may be localized in sedimented particles and in supernatant which is assumed to represent ground plasm of the cell.

Components of the electron transport mechanism, including cytochromes and flavin pigments, cytochrome oxidase, DPN-cytochrome c reductase, and DPNH (reduced DPN) oxidase are present in large

part, and possibly almost exclusively, in the submicroscopic particulates. The enzymes functional in the tricarboxylic cycle. however, are found to be divided between sedimentable particles and the ground substance of the cell. Of these, succinic dehvdrogenase occurs almost exclusively in the granules, and 90 per cent or more of the total activity of the cell may be so accounted for. Malic dehydrogenase is found in both granules and supernatant, while aconitase and isocitric dehydrogenase occur exclusively in the supernatant. The localization of α ketoglutaric dehydrogenase is uncertain. Pyruvic dehydrogenase is also found in the supernatant, but the system linking it to O₂ is in the granules. In the catalysis of some of the physiological activities, then, there is a synergistic relation between the granules and the ground plasm of the cell.

The submicroscopic granules emerge as biochemically complex structures capable of carrying out electron transport as well as the oxidation of organic acids at rates substantially equivalent to those reached by the intact cell. It follows that the components, including cofactors, of the catalytic systems are not only present, but present in physiologically available form, and the individual activities are oriented to give an organization having maximal activity. Thus the submicroscopic particle is an integral basic unit of the cell structure and organization, with a firmly established role in oxidative phosphorylation, 16 and is perhaps the bacterial equivalent of the mitochondrion of larger animal cells. Quantitative considerations suggest that the granules may be enzymatically heterogeneous. Assuming a diameter of 20 m μ , a specific gravity of 1.2, and protein to about one-third of the wet weight, such a particle could contain about 14 protein molecules with an average molecular weight of 35,000. It is difficult to reconcile such an estimate with the known presence of a complete electron transport mechanism and succinoxidase system. If there are enzymatically different kinds of granules, they are not separable by differential centrifugation.

COLONIAL MORPHOLOGY OF BACTERIA

When bacteria are grown on the surface of a nutrient medium solidified with agar or

gelatin, the proliferating cells remain approximately fixed in position and form masses of many millions of cells that are visible to the naked eye. The colonies so formed range from a minute, barely visible size to masses several millimeters in diameter. They have characteristics, not only of size, but also shape and texture, and in some instances color, which, while dependent upon the nature of the culture medium and the conditions of incubation to an appreciable degree, under controlled conditions are constant and often of considerable differential value. Colonial morphology is, then, one of the morphological characteristics of bacteria, and one that is indispensable in primary isolation.

The size of bacterial colonies is, assuming favorable cultural conditions, quite uniform within species or type. Streptococcus colonies are, for example, relatively small, 1 mm. or less in diameter, as are those of most types of the closely related pneumococcus, while those of staphylococcus and enteric bacilli are somewhat larger, and those of Bacillus species may be several millimeters in diameter. Minute, dwarf, or D, colonies, much smaller than usually observed, may occur but represent a variant not usually encountered.

The shape of the colony is determined by its edge and by its thickness. The edge may be smooth, or it may be irregular and serrated to a greater or lesser degree. When the thickness is much greater in the center,

diminishing uniformly to the edge, the colony is said to be raised, occasionally so much so as to approach a hemispherical form. Or it may be relatively uniform so that the colony appears to be little more than a disk on the surface of the medium.

The consistency and texture of the mass of cells are also distinguishing features of colonial morphology. The former ranges from the extreme of a dry, friable nature, such that when the colony is touched with a sterile wire it can be pushed about on the surface of the medium, to a viscous consistency so that the cell mass clings to the wire with which it is touched, stringing away from the colony when it is withdrawn. The surface of the growth may be uniformly smooth and glistening, or it may be striated with indentations, radial or concentric, continuous or broken. On examination with transmitted light, the cell mass may appear as amorphous or granular in texture and varies from almost complete translucency, with perhaps a bluish cast, through varying degrees of opalescence to a white or yellowish opacity.

Pigmentation is more common among the saprophytic bacteria, and the cell mass may appear red, orange, yellow, etc., because of the presence of carotinoid pigments, and green in the case of some of the photosynthetic bacteria containing bacteriochlorin. Of the pathogens, the only important pigmented form is *Staphylococcus aureus*, whose colonies are a golden-yellow in color.

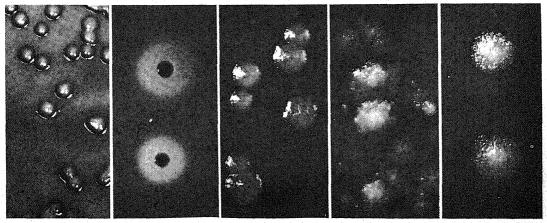


Figure 21. Morphological types of bacterial colonies. From left to right, the raised, smooth, viscous colonies of the gonococcus on chocolate agar; β -hemolytic streptococcus colonies on blood agar showing the cleared zones of hemolysis and the slightly matt, slightly irregular edge of the typical colony; colonies of the typhoid bacillus on nutrient agar, showing the typical irregular edge (maple leaf appearance) and irregular but smooth surface; colonies of the tubercle bacillus on Löwenstein's medium showing the characteristic roughened appearance; and colonies of the anthrax bacillus on nutrient agar in the typically rough, virulent form.

Pigmentation is not apparent in the individual cells for the pigment occurs in intracellular granules too small to be resolved with visible light.

Since these characteristics occur in varying degree and combination from one kind of bacterium to another, colonial appearance is often quite characteristic, and kinds of bacteria may be differentiated from others in mixed or contaminated cultures. Differentiation on the basis of colonial morphology has hardly more than tentative status, however, and detailed study of the physiological and immunological characteristics of a bacterium is ordinarily required for identification.

Colonial morphology is analogous to a statistic in that it is derived from the individual cells but is a characteristic of the cell mass. Thus, pigmentation is apparent not in the individual cell but in the colony; the vicous consistency of the colony derives from the capsular substance of heavily encapsulated bacteria; the coiled texture of Bacillus colonies is a consequence of the tendency of the cells to remain attached end to end and form filaments; actively motile bacteria, such as Proteus, actually move or swarm over the surface of the medium to give a continuous film of growth, etc. In this way colonial morphology may be associated with other significant characteristics of the bacterium, for example, among the pathogenic bacteria virulence may be associated with capsule formation, and when this is true the colony of the virulent form is smooth in appearance and texture, and that of the avirulent form is rough (Chap. Seven).

Colonial characteristics may be accentuated or induced by cultivation of the bacterium on so-called differential culture mediums which exploit relevant physiological properties. When bacteria are grown on an agar medium containing defibrinated blood, areas of greenish discoloration or clear zones in the red opaque medium appear aound the colonies of bacteria which form hemolysins which lyse the erythrocytes and metabolize the hemoglobin to greenish or colorless compounds; such bacteria are said to be α - or β -hemolytic respectively. The ability of diphtheria bacilli to reduce tellurium salts gives rise to black colonies on tellurite differential mediums. Similarly, a sugar such as lactose may be incorporated in the medium together with an indicator such as Schiff's reagent or an acid-base indicator, so that the bacteria fermenting the sugar restore the color to the decolorized fuchsin and the colonies are red or are colored by the indicator because of the local concentration of acidity in the colony. Colonial morphology on such mediums is almost invariably a special case in that the medium is devised to enhance or induce a colonial appearance characteristic of the bacterium in question in order to facilitate its isolation from other bacteria in mixed culture.

The Morphology of Rickettsiae and Viruses

The microorganisms known as rickettsiae and viruses are set apart from the true bacteria, the Eubacteriales, on a physiological rather than a morphological basis. With rare exceptions, the bacteria are not primarily intracellular parasites, and are able to proliferate on lifeless nutrient mediums. In contrast, the rickettsiae and viruses grow only in close association with living ' host cells and are found within the cytoplasm or nucleus. While the largest of these forms link them in continuous series with the smallest bacteria, with progessive decrease in size these agents become distinct both morphologically and in chemical composition.

THE MORPHOLOGY OF RICKETTSIAE

The rickettsiae are morphologically similar to the smallest bacteria. They are coccobacillary to bacillary in form, $0.3~\mu \times 0.3$ to $0.5~\mu$ and occasionally as long as $2~\mu$, and overlap in size range minute bacteria such as Pasteurella tularensis and Bartonella. They are, therefore, demonstrable with the light microscope. They are uniformly gramnegative and stain poorly or not at all unless the dye solution is buffered and mordants are used. In smears and tissue sections they stain purple with Giemsa. In smears stained

by Macchiavello's method (buffered fuchsin, citric acid solution, and methylene blue counterstain) the rickettsiae are red and tissue cells blue, and the buffered methylene blue-safranine counterstain method of Castaneda stains them light blue on a pink background. They also may be stained with diluted Wright's stain following methanol fixation.

They occur singly and in pairs, and most commonly in dense intracellular masses, rickettsiae of the typhus group characteristically in the cytoplasm and those of the spotted fever group in the nucleus of the host cells, particularly those of mesothelial origin which line the serous cavities. They are nonmotile and do not form spores.

The protoplasm stains homogeneously, but Feulgen-positive granules similar to those found in bacteria occur, and both DNA and RNA are present in the cell. In electron micrographs a capsular outer layer, limiting membrane, and electron-dense granules are apparent. In thin sections of Coxiella burnetii the limiting membrane is 5 to 10 m μ thick, and appears to resemble closely the cell wall of bacteria in morphological and chemical properties.^{2, 91} The cell contains a relatively large electron-dense

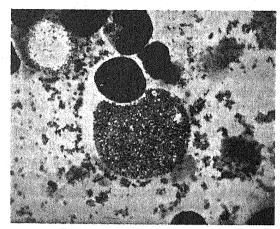


Figure 22. Rickettsiae of Q fever in a smear of skin exudate. Note the macrophage packed with enormous numbers of rickettsiae. × 1125.

body in addition to granules.¹⁰² On treatment of typhus rickettsiae with ether to liberate soluble antigen, the capsular substance is removed and probably represents this antigen.

While the structure of the rickettsiae has not been subjected to as intensive study as has that of the bacterial cell, it seems clear that these microorganisms are closely similar to bacteria, and their physiological char-

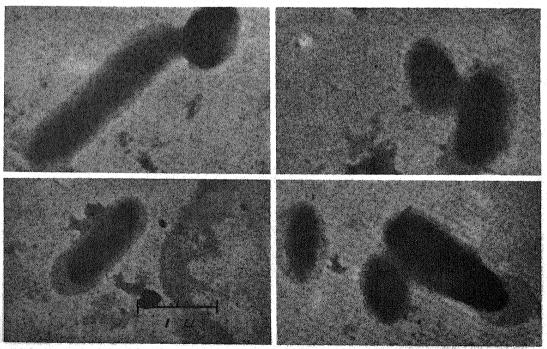


Figure 23. Electron micrographs of typhus rickettsiae from egg yolk culture. Note the variability in size, ranging from coccobacillary to bacillary forms, and the capsular material surrounding the individual cells. (Lilly Research Laboratories.)

acter is not reflected in significant differences in morphology. Since they are not cultivable on lifeless mediums, they have no colonial morphology to correspond to that of the bacteria, but the closely packed aggregates in which they occur within the host cell are suggestive of the viral inclusion body (see below).

While the rickettsiae are incapable of proliferation apart from the host cell, they contain both oxidative enzymes and transaminases. The typhus rickettsiae metabolize glutamate to oxalacetate by way of the scheme

 $\begin{array}{c} \text{glutamate} \rightarrow \text{ketoglutarate} \rightarrow \text{succinate} \rightarrow \\ \text{fumarate} \rightarrow \text{malate} \rightarrow \text{oxalacetate} \end{array}$

and both this mechanism and that of transamination between glutamate and oxalacetate are closely similar to, if not identical with, those found in bacteria (Chap. 5) and higher animals, but these forms lack the enzymatic equipment necessary for independent existence.

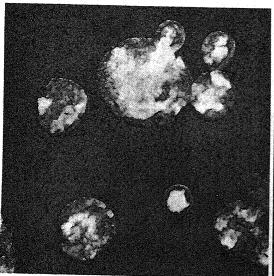
THE PSITTACOSIS— LYMPHOGRANULOMA VENEREUM ORGANISMS^{74, 75}

These agents were long considered to be viruses because of their dependence upon

host cells for multiplication, and cultivation in vitro only in tissue culture. The group includes the causative agents of disease in man, such as lymphogranuloma venereum and trachoma, psittacosis and ornithosis in birds and transmissible to man, and those producing pneumonitis in rodents and diseases of domestic animals such as bovine enteritis and encephalitis.

It has become increasingly clear that these agents are to be distinguished from the viruses, or "true viruses." They contain both DNA and RNA, they are enclosed within a cell wall which is closely similar in its properties to those of bacteria, binary fission appears to be a part of the growth cycle, and the diseases they produce are susceptible to chemotherapy with sulfonamides and many antibiotics. Sensitivity to chemotherapeutic agents is taken to indicate some degree of metabolic independence, and it has, in fact, been shown that these organisms synthesize folic acids and exhibit certain nutritional requirements separable from those of the host cells in which they are cultured.

The morphological character of these agents is complicated by the occurrence of virus particles of two sizes. One, the elementary body, is a sphere about 250 m μ in diameter. In electron micrographs these particles appear as collapsed sacs containing a dense central body apparent as a raised



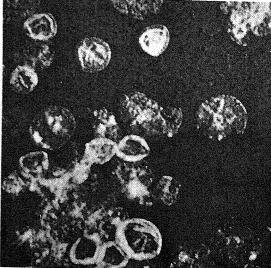


Figure 24. Morphology of microorganisms of the psittacosis group. Left, chromium-shadowed air-dried preparation of the meningopneumonitis agent. The two kinds of particles, relatively large with no central body and relatively small with a dense central body, are apparent. × 20,000. Right, cell walls of deoxycholate-trypsin-treated meningopneumonitis agent, air-dried and chromium-shadowed. × 12,000. (Moulder.)

area in the flattened membrane when the preparation is shadow-cast, and larger, 300 to 450 m μ in diameter, because of the flattening. These particles are taken to represent the infectious agent, probably a resting stage, although a single particle is not sufficient to produce infection.

On inoculation into susceptible tissue, larger bodies known as initial bodies appear after some hours. They are 650 to 800 m μ in diameter and are assumed to be swollen elementary bodies. The structure of these particles is unknown. Still larger bodies, sacs or vesicles, may be found in infected cells and tissues, but these have no distinctive morphology.

THE MORPHOLOGY OF VIRUSES

The viruses, or "true viruses," are set apart from the bacteria, not only because of the exceedingly small size of some of them, but more precisely by their content of only one kind of nucleic acid, DNA or RNA but apparently not both, by their reproduction solely through their nucleic acid, and by the inability of the virus particle to grow or undergo binary fission.

Inclusion bodies. They are replicated only within the host cell, utilizing the intracellular synthetic mechanisms of the cell for the synthesis of viral substance. Virus particles may often, though not invariably, be found in masses or aggregates within the cell, demonstrable by light microscopy and known as inclusion bodies.

Inclusion bodies are divided into two groups on the basis of their location within the cell. The inclusion bodies of the pox group of diseases (variola, vaccinia, fowl pox, etc.), myxomatosis, etc., are found in the cytoplasm of the infected cell. Those associated with other virus diseases are characteristically intranuclear, and are of two kinds. One, designated type A, is a relatively large body which disrupts the structure of the nucleus and is found in cells infected with viruses such as herpes, varicella, and pseudorabies. The other, type B, found in a few virus diseases including poliomyelitis, is much smaller and occurs in an otherwise normal-appearing nucleus.

In some instances inclusion bodies have been named after the workers who des-

cribed them, such as the Guarnieri bodies of variola and vaccinia, and the Negri bodies of rabies. Their presence contributes significantly to the characteristic histopathology of a number of virus diseases. For example, while the rabies virus produces inflammatory and degenerative changes in the central nervous system, especially in the spinal ganglia, the disease may be diagnosed with confidence only by the demonstration of Negri bodies present in the cytoplasm of the large ganglion cells in stained impression smears or tissue sections, preferably of the hippocampus major or cerebellum.*

In at least some instances cytoplasmic inclusion bodies are aggregates of infectious units or virus particles and may be considered as analogous to the bacterial colony. This was unequivocally demonstrated, first with fowl pox, in the now classic study of Woodruff and Goodpasture. When the inclusion body is teased apart, it breaks up into minute granules, 200 to 300 m μ in diameter in the case of the poxviruses and those of the psittacosis group, that may be shown to be the infectious unit of the virus. Various names have been given them in the past such as the Paschen bodies of variola and vaccinia, LCL† or initial bodies of psittacosis, or, more generally, elementary bodies. Such terms, with the possible exception of the last, have tended to drop out of common usage, and the infectious virus unit is commonly called the virus particle, or simply virus.

In other virus diseases such aggregates of infectious units are not demonstrable, and the virus particles are liberated by dissolution of the host cell structure, or in the absence of degeneration are shed into the environment in some other way, in some instances possibly by a process of extrusion.

It is not established that all inclusion bodies found in virus diseases are aggregates of virus. On the contrary, the nature of many inclusion bodies, and intranuclear inclusions in particular, is open to question.

The virus particle. As indicated earlier, the virus particle is extremely small, these

^{*}Failure to find Negri bodies cannot be taken as evidence of the absence of infection with rabies virus. The laboratory diagnosis of this and other virus diseases is described specifically in connection with the diseases.

[†]So-called because they were described independently, and practically simultaneously (1930), by Levinthal in Germany, Coles in England, and Lillie in the United States,

microorganisms ranging in size from the largest, the poxviruses, which are about 200×300 m μ and barely resolvable by light microscopy, to the smallest, such as poliovirus, which are about 25 mµ in diameter. The size range is thus about twentyfold, and the size of the mature virus particle is quite uniform, more so than that of the bacteria which grow as individuals and multiply by binary fission. The particles occur in various shapes, including the brick shape of the poxviruses, the rod-shaped forms of a number of the plant viruses, and the more elaborate tailed structure of bacterial viruses, but a great many appear as approximate spheres. In general, the viruses are characterized in terms of chemical composition and physical structure.

Chemical composition.⁵² It has been possible to prepare a great many viruses in sufficiently purified form to allow chemical analysis with some confidence. The viral particle contains nucleic acid, to perhaps 4 to 6 per cent, which is either DNA or RNA but not both. The presence of only one kind of nucleic acid sets the viruses sharply apart from other organisms, and the occurrence of one or the other type of nucleic acid provides the basis of a primary separation of the viruses into two groups, i.e., the DNA viruses and the RNA viruses.

The viral protein is grossly conventional in composition, or tends to show a preponderance of dicarboxylic amino acid residues in contrast to the basic protamineand histone-containing proteins making up the protein moiety of the usual nucleoprotein. Carbohydrate appears to be a viral constituent of secondary importance, though it may lend immunological specificity to viral antigens. The lipid content of viruses varies widely, ranging from 1 to 2 per cent in papilloma virus to 50 per cent reported to occur in equine encephalomyelitis virus, with viruses such as influenza and herpes falling in between with lipid contents of about 25 per cent. The presence of etherextractable lipid may or may not be associated with activity. In general, viruses are either ether-sensitive, i.e., are inactivated by extraction with ethyl ether, or etherresistant, and this property makes possible a useful separation of the viruses into the two groups.

<u>Structure</u>. The developments in electron microscopy, with continued improvement in fixing and staining methods, and the devising of techniques such as negative staining,⁴³ coupled with x-ray diffraction studies, have made possible the elucidation of at least the major elements in the physical structure of the virus particle.

In a general way, virus particles consist of a central aggregate of nucleic acid surrounded by a protein coat, and in some cases an outer membrane which may be single or double. A terminology of the differentiable components of the particle has become generally accepted²² and is most efficiently described as definitions: The protein shell

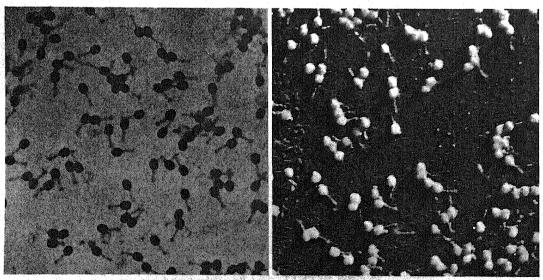


Figure 25. Electron micrographs of coliphage. The plate on the left shows untreated phage, and that on the right a similar preparation shadow-cast with chromium. (Sharp, Taylor, Hook, and Beard.)

surrounding the central core of nucleic acid is known as a *capsid*. The capsid is made up of *structural units* which are assembled in morphological aggregates known as *capsomeres*. The particle made up of nucleic acid enclosed within a capsid is designated a *nucleocapsid*. The outer membrane, which may or may not be present, is the *envelope*. The mature virus particle is a *virion* which may be either a nucleocapsid or a nucleocapsid enclosed in an envelope.

Information concerning the physical organization of nucleic acid in the central core is scant. In some viruses, such as poxviruses, the nucleic acid appears to occur as nucleoprotein, while in others, such as herpesvirus, as nucleic acid alone. Available evidence suggests also that the nucleic acid is present as one molecule, and that this is folded to produce packets in situ.

Of the other morphological characteristics of the virus particle, the structure of the capsid has been of greatest interest. 61, 84, 117 Some viruses, such as adenoviruses, may be found in orderly arrays of virus particles in masses within the host cell, and others, such as poliovirus, not only show such arrays but have been prepared in crystalline form. Such orderly arrangements reflect a symmetrical structure of the capsid and orientation of its component capsomeres. As Crick and Watson²⁶ pointed out in their prediction (1956) of a polyhedral structure of small viruses, the coding of small identical protein molecules would constitute a highly efficient use of the limited genetic information contained in the viral nucleic acid.

Symmetry. Theoretical considerations²³ indicate that the most efficient designs of a protein shell are helical tubes and icosahedral shells, the latter embodying the same principles of geometric design as those of the geodesic dome structures of Buckminster Fuller. It has been found that many, though not all, viruses show helical or cubic symmetry; obvious exceptions include the poxviruses and bacterial viruses.

Of the viruses showing helical symmetry, many are plant or insect viruses, and of these perhaps the best known is the tobacco mosaic virus. As described below, the capsid of this virus is a hollow tube of protein in the form of a helix, and made up of 16½ subunits per turn. Certain animal viruses, notably myxoviruses, also have helical symmetry though this is less overtly obvious

since the particles are roughly spherical in shape. The helical component of influenza virus, for example, appears to be tightly wound or packed within an envelope. It is not seen in the usual preparations, but in partially disrupted particles is found in whorls and parallel strands.

The usual type of viral cubic symmetry is icosahedral, and many of the animal viruses, including poliovirus, herpesvirus, reovirus, adenovirus, and polyoma virus, fall into this group. Such symmetry is the basis of the structure of the capsid, but the virus particles need not conform precisely to this shape. Such viruses fall into classes with respect to the number of capsomeres making up the capsid, viz., 12, 32, 42, 92, 162, 252, and 812. Formulas have been presented for the calculation of the number of capsomeres, and the general formula of Wildy and Horne is $10x(n-1)^2+2$ where x may have a value of 1 or 3, and n is the number of capsomeres between and including those on adjacent five-fold symmetry axes.²³ There is reason to believe that the icosahedral capsids are constructed with two kinds of capsomeres, the one pentagonal and containing five asymmetric units, and the other hexagonal and made up of six asymmetric units. For example, adenovirus types 2 and 5 have 252 capsomeres, of which 12 pentagonal capsomeres are on the vertices, and 240 hexagonal capsomeres on the edges and faces.

In addition to the capsid, and envelope if present, other structures, demonstrable by negative staining for the most part, may be found in a number of kinds of virus particles. The Poxvirus particles, for example, have been found to contain diagonal tubular structures in irregular pattern, and influenza virus particles have a surface (envelope) structure characterized by regular projections. The nature of these and other components of the virus particle are as yet only poorly understood in terms of chemical composition, antigenicity, function, etc. 79, 113, 118

THE BACTERIAL VIRUSES (BACTERIOPHAGES)1, 3, 20, 99, 100

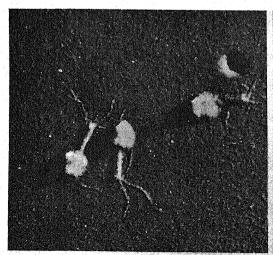
Transmissible lysis of bacteria was discovered by Twort in 1915 and rediscovered by d'Herelle in 1917 and for many years was known as the Twort-d'Herelle phenom-

enon. The agent is present in bacteria-free filtrates of lysed cultures of bacteria, and the addition of such filtrate to a fresh, growing culture results, after a suitable period of incubation, in lysis manifested as a clearing of a broth culture, or a cleared zone, or plaque, in a uniform film of bacterial growth on the surface of an agar culture medium. This phenomenon is a consequence of the activity of viral agents parasitic on bacterial cells. There are very many of these bacterial viruses, or bacteriophages, some broadly specific and others narrowly specific, even within species, for the host bacteria.

Since the bacterial viruses are readily manipulated in the laboratory, they have served as prototypes in the investigation of the nature of viruses, although it does not follow that they are directly comparable in all respects with animal and plant viruses. Consequently, they are better known than most of the other viruses, and of the bacterial viruses a group parasitic on coliform bacilli have been by far the most intensively investigated. There are seven of these "T" strains in all, of which T2, T4, and T6, the "T-even" phages, constitute one serological group, T_3 and T_7 another, while T_1 and T_5 are unrelated to one another or the other T-coliphages. All of these are tadpoleshaped, as are other phages which have been examined critically, but the tails of T₃ and T₇ are very short. Phages parasitic on other bacteria, including staphylococci, streptococci, and mycobacteria, also appear to have this same general type of morphology. 12

In electron micrographs of freeze-dried preparations of the T-phages, the heads are polyhedral, almost crystalline, in form and, when observed either parallel or perpendicular to the tail, have six relatively linear sides. The heads of the T-even phages are elongated hexagons, 65×95 m μ , while the other T-phages have hexagonal heads, ranging in size (diameter) from 47 mu for T₃ and T_7 , through 50 m μ for T_2 to 65 m μ for T₅. The tails vary in size and proportion; those of the T-even phages are $100 \times 25 \text{ m}\mu$, those of T_1 and T_5 are more slender, $150 \times$ 10 m μ and 170 × 10 m μ respectively, while those of T3 and T7 are stubby and measure 15×10 m μ . The tail is not an organ of locomotion, for it does not contribute to the rate of diffusion of the phage, i.e., while the rate of adsorption on bacterial cells is proportional to the cell concentration, it is considerably less than the calculated collision rate and therefore not consistent with the assumption that the bacteriophages are actively motile.

The phages are DNA viruses, though RNA phages have been described. ¹²¹ Detailed study has made it clear that the phage particle is a differentiated, relatively complex structure. The head portion is made up of DNA, ⁸ making up as much as 40 per cent of the virus particle as noted above, enclosed in an envelope or membrane of protein. The latter apparently has some function as an



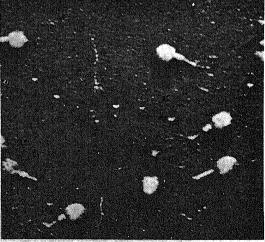


Figure 26. The structure of the distal portion of the tail of the T-even coliphages. Left, T₄ frozen and thawed to free the protein strands. × 100,000. (Williams.) Right, the protruding tail spike of T₂ coliphage apparent after removal of the distal tail protein by treatment with peroxide. × 100,000. (Kosloff.)

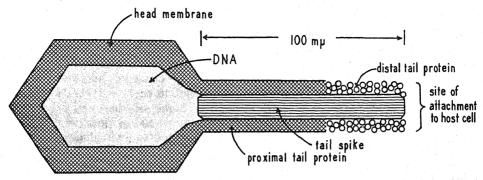


Figure 27. Schematic representation of the differentiated structure of coliphages of the T-even series.

osmotic barrier for, when the particle is subjected to osmotic shock, the DNA leaks out to leave an empty shell.²⁹

The tail structure is also complex, the component parts playing different roles in the first stage of the reproductive cycle in which the phage particle is adsorbed at the tip of the tail onto the surface of the bacterial host cell (Chap. Four). The tail appears to be a tubular structure closed by a plug or tail spike. In the T-even and certain typhoid bacillus phages, the tail sheath is contractile, but in other phages it is not. The distal portion of the tubular structure is differentiated from the proximal portion in that the last few m μ are made up of protein strands held together by a thiol ester linkage. It may be depolymerized by treatment with Zn⁺⁺ or Cd⁺⁺, and is depolymerized following adsorption onto the host cell, in both instances leaving a portion of the tail spike uncovered. In addition, the extreme tip of the tail is differentiated, apparently with respect to the distribution of positively charged amino groups that play an essential part in the initial absorption.

Serological studies have shown that at least two immunologically distinct proteins are present in the phage particle, the one in the head membrane and the other in the tail; antibody to the tail portion is that which neutralizes phage infectivity. It is clear that at least four distinguishable proteins are present, viz., that of the head membrane, that of the proximal portion of the tail, that of the distal portion of the tail, and that of the tail spike.

The formation of areas of bacteriophage

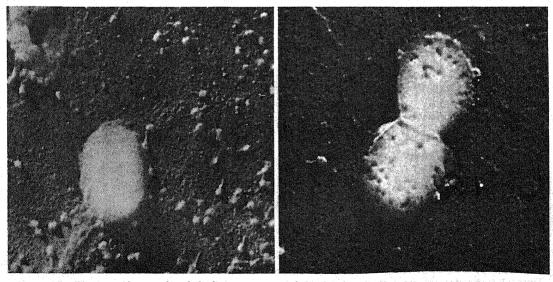


Figure 28. Electron micrographs of shadow-cast preparations showing the lysis of coliform bacilli by coliphage T_4 . Left, an intact bacillus surrounded by phage particles; right, a bacillus about to undergo fission from which the cell membrane has begun to peel away in the initial stage of lysis. \times 20,000. (Wyckoff.)

lysis in the confluent growth of susceptible bacteria on the surface of agar mediums has been noted above. When these are discrete, they are analogous to bacterial colonies in that it is a reasonable assumption that the area is a result of the proliferation of a single phage particle and consists of the progeny of that particle just as the bacterial colony is made up of the progeny of a single viable cell. Such discrete areas of phage lysis are known as plaques. Thus phages may be isolated in "pure culture" by streaking an agar medium previously inoculated sufficiently heavily with susceptible bacteria to give confluent bacterial growth, and the plaques may be picked and "subcultured." The occurrence of phage plaques also makes available an additional morphological character, that of plaque morphology.

While there are not as many variables, and therefore combinations of characteristics, in bacteriophage plaques as in bacterial colonies, the plaques have a distinctive character of differential value. There are, for example, "large plaque" phages, "small plaque" phages, and "minute plaque" phages. Another plaque type is the tu, or turbid halo, type characterized by a clear center surrounded by a diffuse cloudy zone. Or the margin of the plaque may be sharply defined or hazy. These plaque characteristics have the same validity as those of bacterial colonial morphology and may be regarded as phenotypic expressions of genetic character.

ANIMAL VIRUSES4, 18

The animal viruses fall into a number of differentiable morphological types observable by electron microscopy, including examination of negatively stained preparations. Some of these may be considered briefly here, and in more detail in later chapters devoted to individual or groups of viruses.

Poxviruses. The viruses making up the pox group are relatively homogeneous morphologically. The group includes the causative agents of diseases of man such as variola, vaccinia, molluscum contagiosum, and diseases of lower animals such as myxoma and fibroma of rabbits, fowl plague, and mouse pox.

The virus particles are brick-shaped and relatively large, 200×300 m μ , though some may be somewhat smaller. In electron micrographs a dense central portion is apparent. Digestion with pepsin accentuates the appearance of the central body and shows a limiting membrane. Subsequent treatment with deoxyribonuclease results in the dissolution of the central body, leaving the virus particle as an empty membrane. The structural similarities to the bacteria, *i.e.*, a central mass of DNA surrounded by "viroplasm" and enclosed within a single or double limiting membrane, have been noted by many workers.

Myxoviruses. 42 The viruses of this group are RNA viruses, are generally spherical

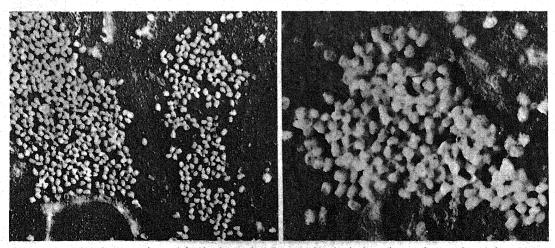


Figure 29. An electron micrograph of thin section through the chorioaliantoic membrane of a 10-day chicken embryo 24 hours after inoculation with vaccinia. The numerous elementary bodies can be seen to possess a cuboidal or cylindrical shape. Left, × 4500; right, × 12,500. (Wyckoff.)

in shape, have helical symmetry, and are ether-sensitive. The nucleoprotein coil is enclosed in an envelope in which host cell constituents, particularly lipids, are found. The outer structure of the particle is readily deformed, leading to variability in observed size. The term myxovirus refers to an affinity for mucinous substances, such as that found on the surface of erythrocytes, where it is functional in viral hemagglutination, and these viruses have an enzyme, neuraminidase, which depolymerizes the mucinous substrate.

These viruses fall into two groups, designated group I and group II, or subgroup I and subgroup II. The viruses of group I are 80 to 120 m μ in diameter, and the nucleoprotein coil has a diameter of about 9 m μ . Group II contains the larger viruses, 150 to 250 m μ in diameter, in which the nucleoprotein coil has a diameter of about 18 m μ . There tends to be some morphological variation, as in the appearance of influenza virus in filaments, and the varied shapes assumed by Newcastle disease virus in particular.

Group I includes the human influenza viruses types A, B, and C, swine influenza virus, and fowl plague virus, while group II is made up of Newcastle disease virus, mumps virus, the parainfluenza viruses, and the simian viruses SV5 and SV41. Some other viruses, such as the Rous sarcoma

virus, measles virus, rinderpest virus, and canine distemper virus, are closely similar morphologically to the accepted myxoviruses.

Herpesviruses. The viruses of this group are DNA viruses, in which the DNA may be double-stranded. They have cubic symmetry, with 162 capsomeres making up the icosahedral capsid, and are enclosed in a double envelope, and the mature virus particle is spherical and 120 to 130 m μ in diameter. The herpesviruses are ether-sensitive.

The group includes the viruses of herpes simplex of man, and B virus, pseudorabies virus, and virus III occurring in lower animals. The varicella-zoster (chickenpoxherpes zoster) virus is somewhat larger, 150 to 200 m μ , and while once thought to be related to the poxviruses, is now considered to be a herpesvirus. There are a number of morphologically similar viruses, such as cytomegalovirus of man, and viruses producing respiratory disease in lower animals, which are regarded as herpes-like, and considered by some to be herpesviruses.

Adenoviruses. These viruses are DNA viruses having an apparent filamentous structure in the nucleoid. They show cubic symmetry, with 162 or 212 capsomeres making up the icosahedral capsid, and are approximate spheres having a diameter of 70 to 80 m μ . The virus particles often appear in orderly arrangement in crystalline-

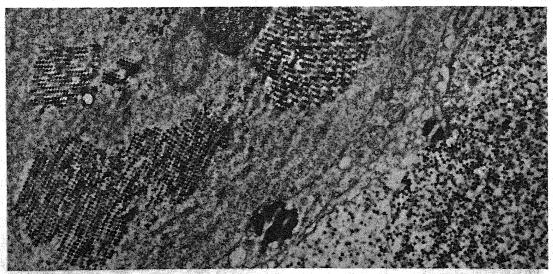


Figure 30. Adenovirus in two adjacent cells. In the cell on the right the virus particles are dispersed at random, but in the cell on the left they occur in ordered arrays, and the latter aggregates may be seen with the light microscope within infected cells. The two kinds of virus particles, the one more electron-dense than the other, are clearly apparent. × 11,550. (Councilman Morgan.)

like cytoplasmic inclusion bodies in infected cells. These viruses are ether-stable.

The adenoviruses of man are also known as the APC (adenoidal-pharyngeal-conjunctival) viruses after the kinds of disease they produce, and are separable into numbered serological types. Closely similar viruses are of simian, bovine, murine, and avian origin. The avian adenovirus is often referred to as GAL (gallus-adenovirus-like) virus. It has been suggested that infectious canine hepatitis virus is an adenovirus.

Picornaviruses.⁸² This is a group of RNA viruses of very small size, 22 to 27 m μ in diameter, which has given rise to the name: pico (small), RNA, virus. The picornaviruses have cubical symmetry, and possibly 42 capsomeres make up the icosahedral capsid. Their content of nucleic acid is relatively high, 25 to 30 per cent, and they are ether-resistant.

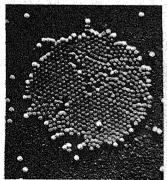
The viruses of human disease in this group include those known as the enteroviruses, i.e., polioviruses, the Coxsackie viruses, the ECHO (enteric cytopathic human orphan) viruses, and the rhinoviruses which are causative agents of the common cold. Poliovirus and Coxsackie type A viruses have been prepared in crystalline form. A number of viruses which are the etiologic agents of disease in lower animals also fall within the group, i.e., the virus of foot-and-mouth disease of cattle, the encephalomyocarditis virus, and that causing Teschen disease of swine.

Papovaviruses. These are DNA viruses, containing double-stranded nucleic acid, and are considered to be oncogenic, or poten-

tially oncogenic. The name derives from papilloma, polyoma, and vacuolating agent, and was suggested by Melnick to meet with general acceptance. In general, these viruses are relatively small, 30 to 50 m μ in diameter, have cubic symmetry with 42, 60, or 92 capsomeres making up the capsid, and have no envelope, but there is some variation, or at least uncertainty, within the group.

The etiologic agent of infectious warts in man is a member of this group, and has been reported to be 45 m μ in diameter, with 42 capsomeres in the capsid, and tends to form crystalline-like aggregates within the infected cells. The rabbit papilloma virus of Shope is also about 45 m μ in diameter, and 42, 60, and 92 capsomeres have been reported. There are a number of other papilloma viruses of lower animals which are not well known but considered to be papovaviruses. The polyoma virus, an oncogenic virus with less marked host specificity, is about 45 m μ in diameter also, and the number of capsomeres has been variously reported as 42 and 92. The so-called vacuolating virus of simian origin, SV40, is also a member of this group, measures 40 mµ in diameter, and has 42 capsomeres making up the capsid.

Arboviruses. The viruses of this group have the common property of being arthropod-borne, hence the name, but in general are much less well known than many other viruses, and may be a heterogeneous group and include viruses which may be found to have other affinities. They are probably all RNA viruses, are ether-labile, and the nucleocapsid is enclosed in a membrane.



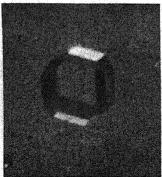




Figure 31. Poliovirus. Left, purified virus, showing the spherical form and tendency of the virus particles to pact together. \times 8000. Center, a crystal of poliovirus. \times 100. Right, the sheared surface of a crystal of the virus, showing the occurrence of the virus particles in orderly arrays. (Schwerdt.)

Their symmetry is uncertain as yet, they are spherical in shape, and they range, from one virus to another, from 20 to over $100 \text{ m}\mu$ in diameter. The majority have been given place names, e.g., eastern, western and Venezuelan equine encephalomyelitis virus, Sindbis virus, Semliki Forest virus, West Nile virus, and Japanese B encephalitis virus, and rarely descriptive names such as Chikungunya virus. There are two types, type A and type B, separable by hemagglutinin character, but apparently not morphologically distinct.

THE INSECT VIRUSES 10, 93, 94, 97, 98

A number of infectious diseases of insects, especially insect larvae, are caused by viruses. The majority of these diseases are characterized by the presence of inclusion bodies in the infected cells, which are made up of virus particles. Sacbrood of bees is exceptional in that inclusion bodies are not found.

Many of these inclusion bodies are polyhedral in shape, ranging in size from 0.5 to 15 μ , but most commonly 3 to 5 μ , and the diseases in which they occur are known as polyhedral disease or polyhedrosis. The best known of these is jaundice of silkworm (Bombyx mori) larvae and has been recognized since the early sixteenth century.

Other insect larvae infected by similar agents are the nun moth, the gypsy moth, and some species of caterpillars.

The other kind of inclusion body is a small oval body, usually less than 1 μ in length, and the associated viruses are sometimes known as the capsule viruses or granulosis viruses. They produce disease in certain caterpillars, cutworms, and butterfly larvae.

The intact inclusion bodies are insoluble in water and are not affected by the processes of bacterial decomposition of the host tissue, so they may be prepared in relatively pure form by centrifugation. They may be dissolved in dilute alkali to liberate the virus particles, and presumably an analogous process occurs in the gut of the insect at the initiation of infection. The inclusion bodies consist of nucleoprotein (DNA type) virus particles embedded in a matrix of protein, which is serologically related to that of the virus particles, and enclosed in a "limiting membrane" of precipitated specific, or closely related, protein. The protein matrix makes up about 95 per cent, and the virus particles 3 to 5 per cent, of the inclusion body.

The particles of the majority of the insect viruses are rod-shaped, 20 to 27×200 to $400 \text{ m}\mu$. It was believed for many years that all the insect viruses were rod-shaped until it was shown that in some instances

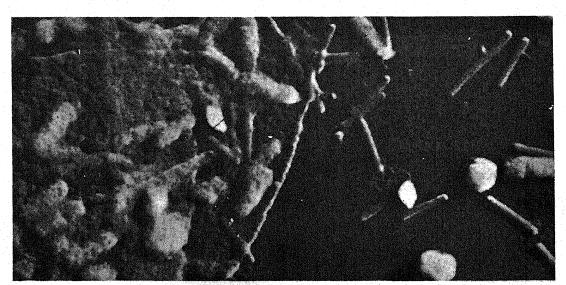


Figure 32. The virus of polyhedral disease of the gypsy moth (*Porthetria dispar*) showing the polyhedral body on the left. The "packets" of rod-shaped virus particles are enclosed within limiting membranes both within the polyhedral inclusion body and outside it, and the rod-shaped particles are those of mature free virus. × 22,000 (Bergold.)

spherical forms, 20 to 40 m μ in diameter, may predominate.

The rod-shaped particles occur in the inclusion body in small aggregates enclosed in a membrane. In the polyhedral inclusion body there may be six to eight individual rods arranged as bundles, while in the smaller oval inclusions single rods predominate, and occasionally two or three may be found within a single membrane. In the intact or disintegrating inclusion body, spheres may be observed occasionally, and there is reason to believe that the rods originate in the spherical bodies, and break down to liberate spheres in a reproductive cycle. Filaments may occur also which are reminiscent of those associated with some of the animal viruses. In its early stages the inclusion body is liquid in consistency, but it becomes more viscous as the protein concentration increases, and finally protein precipitates at the periphery to complete formation of the mature inclusion. In any case, it is probable that the rods represent the resting stage of the majority of these viruses, but in some instances the sphere may be the resting stage.

PLANT VIRUSES⁵⁹

A considerable number of diseases of plants are of virus etiology, and the third group of viruses is made up of these agents, the plant viruses, separable into more than 100 kinds. The diseases they produce include the yellows group of diseases (such as aster yellows); the mosaic diseases of tobacco and acuba; the ring spot diseases of tobacco, potato, and delphinium; the leaf-curl diseases of tobacco, cotton, and sugar beets; the leaf savoy (leaf deformation) diseases of beets and rutabaga; and spotted wilt of tomato.

Almost all of these agents are transmitted by arthropod vectors of the families Aphididae (aphids), Cicadellideae (leaf hoppers), Aleyrodideae (white flies), Coccideae (mealybugs), and Thrysanoptera (thrips). Of these vectors, the aphids are by far the most common, the leaf hoppers less so, and only one plant virus, that of spotted wilt, is transmitted by thrips; the white fly vectors are more common in tropical climates than in temperate climates. While transmission may be mechanical, and usually is for example in aphids, it may also be biological in

that the insect vector is infected, is capable of transmitting the virus only after an incubation period, and may remain infectious for long periods or even for life. In a few instances, such as rice stunt virus, the virus is transmitted from the female to the egg.⁵⁸ Such biological transmission is of particular interest in that it indicates that the range of hosts capable of supporting growth of the virus extends from the plant to animal kingdoms. A number of these viruses have been cultivated in tissue cultures which simulate the insect tissues.³⁵

Some of these viruses have been studied intensively, while others are not so well known. In general, they occur as two morphological types, the one rod-shaped and relatively large and including tobacco mosaic virus (TMV) and cucumber virus, both 300×15 m μ , and the virus of potato-X disease which is a somewhat longer, more slender rod, 430×10 m μ . The remainder of the plant viruses are small spherical or polyhedral particles 15 to 30 m μ in diameter. A number of these viruses, notably TMV and cucumber viruses, and the viruses of bushy stunt of tomato, tobacco necrosis, Southern bean mosaic, and turnip yellow necrosis, have been prepared in crystalline form. These viruses appear to consist of pure, or nearly pure, nucleoprotein. The nucleic acids of the plant viruses are of the RNA type exclusively, in contrast to the bacterial and animal viruses, with nucleic acid contents ranging from 5 to 6 per cent to as high as 40 per cent in the case of tobacco ring-spot virus. Two of the plant viruses, TMV and bushy stunt virus, may be taken, for present purposes, as examples of the better known plant viruses.

Tobacco mosaic virus.^{81,96} This virus, the first to be crystallized, was prepared by Stanley in 1934 as needle-shaped paracrystals. It is a pure nucleoprotein, containing 94 per cent protein and 6 per cent nucleic acid of the RNA type, with a molecular weight of about 5 million.

The infectious unit is a cylinder of protein, 300×15 m μ , with a core of nucleic acid, probably multistranded, about 4 m μ in diameter. The virus may be found in, or broken down to, units of less than this length, and may occur in multiples of it, but the smaller rods do not have virus activity. The rods are deeply grooved and may be packed screw-wise more tightly than the

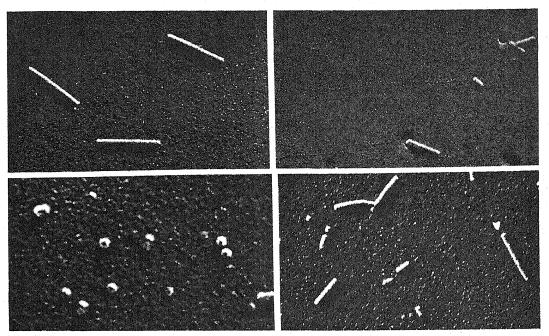


Figure 33. Tobacco mosaic virus. Upper left, the infectious particle of the virus. \times 58,000. Upper right, partially disintegrated virus particles, showing the fine strands of the central core of nucleic acid attached to the fragments. \times 42,000. Lower left, the washer-shaped units of the cylindrical protein structure. \times 115,000. Lower right, "reconstituted" virus, showing the variability in size of the particles so produced. \times 50,000. (Williams.)

diameter of 15 m μ would suggest. The way in which the nucleic acid and protein components are bound is not known, but it is of such a nature that the nucleic acid is not attacked by RNA-ase in the intact particle.

The particle may be broken up by treatment with alkali, sodium dodecyl sulfate, or urea, with separation of protein and nucleic acid, and disintegration of the protein into chemical subunits with a molecular weight of 17,000 to 18,000 in which threonine is the C-terminal amino acid, and the N-terminal is bound in a ring structure. The peptide chain of 145 residues is coiled in the form of a helix so that the subunits have a washer-like form. The minimum size of subunit that can be polymerized to give a rod-shaped structure is, however, larger than this, with a molecular weight of about 100,000.

It has been possible to copolymerize the protein and nucleic acid components after separation in sodium dodecyl sulfate to give rods of the original composition and size and having viral activity. Further, a "hybrid" virus has been prepared by combining nucleic acid from the ribgrass strain of TMV with protein from ordinary TMV to give a virus that produced a disease characteristic of the ribgrass strain and whose progeny contained both ribgrass protein and nucleic acid. The relatively greater importance of the nucleic acid portion of the molecule is further indicated by the production of relatively extensive changes in the protein without affecting viral activity. Thus, activity is not lost when as much as 70 per cent of the free amino groups are acetylated, or all the -SH groups are re-

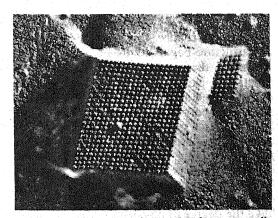


Figure 34. Electron micrograph of an unusually perfect rhombic type crystal of tobacco necrosis virus, prepared by the carbon replica technique. × 42,000. (Labaw and Wyckoff.⁵⁴)

acted with iodine, though inactivation occurs when iodine is substituted in the tyrosine ring. Again, the progeny of such altered virus contain the normal TMV protein component. It is clear that such studies are beginning to approach the goal of some understanding of the nature of viral, or for that matter protoplasmic, replication during the processes of growth.

Bushy stunt virus.²¹ The virus causing bushy stunt of tomato is representative of the smaller spherical plant viruses. It has been crystallized in the form of dodecahedral crystals and is apparently largely, if not entirely, nucleoprotein, but contains considerably more, 16 per cent, nucleic acid than TMV. In electron micrographs of airdried preparations it appears to be spherical in shape and about 30 m μ in diameter. In freeze-dried preparations, it is apparent that the virus particles are polyhedral rather than spheroid in shape and in cross section are usually hexagonal. The virus particles tend to occur in orderly arrays, and the crystals are made up of such arrayed particles. X-ray diffraction studies suggest that the virus particle is made up of 60 subunits with a molecular weight around 125,000.

COMPARATIVE MORPHOLOGY OF MICROORGANISMS

The fungi and animal parasites considered elsewhere are more complex and morphologically conventional than the bacteria in that even the unicellular forms are differentiated into the usual nucleus and other intracellular structures. Beginning with the bacteria, and the blue-green algae not touched upon here, this familiar pattern of cell structure begins to dissolve.

Among the bacteria the existence of a functional, morphologically discrete nucleus becomes nebulous, and its place is perhaps taken by the chromatinic body, containing large amounts of DNA but of no more than equivocal relation to a nucleus. Polyphosphate-containing granules visible with the light microscope occur, but apparently function in the storage of energy in the form of high-energy phosphate bonds, and do not have the degree of physiological competence associated with the mitochondrion of conventional cells. Rather, mitochondrial

function is associated in part with minute intracellular particulates, and quantitative considerations suggest that even these, as individual particles, may not have the metabolic activity they show in the aggregate. The cell wall persists as a supporting structure and may, because of its minor contribution to the osmotic barrier, have survival value, but it may be dispensed with to leave a functional physiological unit, the bacterial protoplast, apparently lacking only the ability to initiate spore formation.

In the size range of the larger viruses the inevitable effects of size become even more apparent in that the differentiated structure that persists serves to house and protect a nucleic acid directive mechanism, and the unit consists of little more than a protective membrane surrounding an electron-dense body consisting, presumably, largely of nucleic acid compounds. Finally, in the simplest viruses there is little or no structure, and the particle consists of naked nucleoprotein that has organizational individuality only indirectly through the directive mechanism it contains and possibly can be considered as "living" only because it can reproduce itself indefinitely in series in a host cell. It is probable that the "living organization" reaches its lower limit at the dimensions of large protein molecules; thus the smallest subunit of TMV that can be polymerized to give an infectious unit has a molecular weight of 100,000, and the subunit of bushy stunt virus has a molecular weight of 125,000. This apparent lower limit in size may be taken to represent that in which the nucleic acid is effectively protected by the protein coat, i.e., that of resting virus. With the demonstration of infectious nucleic acid (Chap. Four), the lower limit may be reduced to that of the size of a macromolecule of polymerized nucleic acid.

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Chapter Four

THE GROWTH OF MICROORGANISMS

The growth of microorganisms is essentially the specific, balanced synthesis of the components of protoplasm from the nutritive substances present in the immediate environment. In addition, the newly synthesized constituents must be assembled and appropriately packaged to yield replicates of the original unit. The specificity of the entire process is dependent upon the operation of directive mechanisms, or genetic controls, which have the unique property of self-replication so that they appear as intrinsic parts of the new units.

This complex process is obviously dependent upon, and affected by, the kinds and concentrations of nutrient substances present in available form and upon a continuous supply of energy required for the endothermic reactions of synthesis. The source of the latter is the coupled oxidation-reduction reactions of respiration, so that, in the broad sense, the nutritive environment also includes oxidizable substrates together with reducible substances functioning as immediate and/or ultimate hydrogen acceptors.

Microbial growth may be approached in a number of ways. The most obvious are the cytological approach, that of the growth of microorganisms as population, and that of the nature of the biochemical processes involved. Such separations are artificial in that they represent no more than relative emphasis on one or another view of the same process but are useful for purposes of discussion. The first two will be considered here, and the last elsewhere (Chap. Five).

THE GROWTH OF BACTERIA

By far the most common method of multiplication of bacteria is that of simple binary fission in which the cell apparently constricts and separates into two daughter cells. Fission occurs in a single plane, at a point indicated by the development of a thickened, equatorial band in the cell wall, at a right angle to the long axis of bacillary and spiral forms. It may occur in one, two, or three planes of the spherical forms, making possible the characteristic morphological groupings described earlier (Chap. Three). Bacilli and spirochetes show some elongation prior to fission. The cocci do not, as a rule, although there may be some increase in the diameter of the cell without significant alteration of its spherical form. The size a single cell must reach before fission is quite constant, although differences in this maximum are associated with the age of the culture (see below).

While cell division is clearly intimately related to the synthesis of cell substance, it may appear to be a distinct process in that, within limits, cell division may be inhibited by failure or inhibition of cell wall synthesis, while other components of the cell substance continue to be formed. One of the effects of penicillin, for example, is an inhibition of cell division of staphylococci, and the individual cells continue to grow without dividing and become greatly enlarged. Cell division may be inhibited in a number of other ways as, for example, by culture in magnesium (Mg⁺⁺) deficient medi-

ums, by treatment with certain organic compounds such as 5-diazouracil, acriflavine, or methylenemelamine, or with ionizing radiation such as x-radiation or α -radiation. When rod-shaped bacteria are treated in this way, they fail to divide and continue to grow as long filaments.

More precise definition of the internal structure of the bacterial cell has made more clear some cytological aspects of cell division. 19, 47 The first step is the formation of a septum, or cell plate, across or slightly oblique to the long axis in the case of bacillary forms, dividing the cell contents into approximately equal portions. In gram-positive bacteria this develops by centripetal growth of the plasma membrane, followed closely by the cell wall. The manner in which this occurs is not entirely clear, viz., whether the plasma membrane grows by extension, or whether its formation is associated with the minute peripheral bodies that appear in electron micrographs of ultrathin sections to move inward with the centripetally growing cell wall. In any case, constriction of the cell occurs, and the two daughter cells separate. In the gram-negative bacteria, growth of the cell wall appears to be general rather than local, followed by constriction prior to cell division.

While bacterial multiplication usually takes place by transverse fission, other kinds of cell division may occur. Some, such as the diphtheria and tubercle bacilli show true branching that is characteristic of some of the higher fungi, and budding, similar to the budding of yeast cells, has been described by some workers. More complex processes occur, such as the formation within the cell of viable granules, or gonidia, which on liberation develop into morphologically typical cells; this is firmly established as the so-called swarmer stage in the development of some soil bacteria, but is not otherwise of common occurrence. Another kind of more complex reproductive process occurs in the Bacteroides and Mycoplasma (Chap. Twenty-seven), in which these structurally fragile cells swell to form large round bodies that then may undergo multipolar germination, with segmentation of outgrowing processes to form daughter cells. All these more complex kinds of reproduction are quite uncommon in that they may occur in only a very few kinds of bacteria with some regularity, or extremely rarely in many kinds of bacteria.

Conjugation between bacterial cells of differing sexuality occurs with transfer of genetic determinants (Chap. Seven) but, like spore formation, it is not considered to be a mechanism of replication *per se*.

The generation time in binary fission, *i.e.*, the time elapsing between cell divisions, is minimal during the exponential growth phase (see below) of the bacterial culture. Certain enteric forms, such as the cholera vibrio and coliform bacilli, are among the most rapidly proliferating bacteria, with generation times of 20 minutes or less, and one marine Pseudomonas species has been found to have a generation time as short as 9.8 minutes.²⁵ More slowly growing bacteria, such as the tubercle bacilli, have generation times of several hours, or even days.⁶⁸

The rapidity of cell division among the bacteria is not unique as sometimes supposed, for embryonic cells of higher forms may multiply equally rapidly. The unique aspect of the rate of bacterial multiplication lies, rather, in the fact that such a short time is required for the bacterial cell to reach full maturity.

THE GROWTH OF BACTERIAL POPULATIONS

For obvious practical reasons bacteria are ordinarily manipulated and studied, not as individuals, but as aggregates made up of very large numbers of cells. Bacterial growth may be considered as the growth of populations of many millions of cells whose characteristics are essentially statistical ones, and the behavior of individual cells is assayed as a frequency.

Growth of bacteria in culture may be considered from a number of points of view, and the methods of its measurement depend upon the purpose at hand. A simple view that is commonly, if tacitly, taken assumes that growth may be described by the reaction:

culture medium $+ O_2 \rightarrow$ bacterial protoplasm + oxidized substance

which proceeds from left to right, and eventually comes to an equilibrium. The reaction velocity constants may be determined by the rate of disappearance of the reacting substances or the rate of appearance of end products.

Although complicated by a variety of side reactions, the assumption of such a relation has been extremely useful in studies on bacterial metabolism. The growth of autotrophic bacteria, for example, is conveniently measured in terms of the conversion of carbonate to organic carbon. A somewhat more mechanistic concept is that of the instability of an inoculated culture medium which, after undergoing a series of complex changes, reaches a stable equilibrium at a lower energy level and provides the basis of studies on the energy metabolism of bacteria.⁷⁶

The most common method of measuring the growth of bacteria in culture is by periodic counting of the number of cells present. This may be a viable count, made by quantititative dilution and plate culture, or as total cells by direct cell count, or indirectly as turbidity translated into numbers with a previously prepared calibration curve. Or the total number of bacteria present may be measured as dry weight.

When all, or practically all, the cells are viable and multiplying by binary fission, the rate of growth is exponential. This potential rate of multiplication may be realized only up to a certain point. As numbers increase in the microcosm of the culture tube,

competition between individual organisms for food stuffs, oxygen, and the like, progressively reduces the opportunity for further growth until a saturating population density is reached.

If no increasingly effective retardation were operative, the potential increase would be expressed by the relation:

$$\frac{dY}{dt} = bY$$

where Y is equal to the number of individuals per unit volume, and b to the rate of growth, or generation time, of the microorganism. When there is a maximal possible cell density, K, this geometric rate of increase is only partially realized, the extent of the realization depending on how near the culture is to its maximum density at any given time. Or, mathematically:

$$\frac{dY}{dt} = bY \frac{K - Y}{K}$$

This is the differential equation of the logistic function:

$$Y = \frac{K}{1 + e^{a - bx}}$$

This function, plotting as a symmetrical S-shaped curve, describes the growth of populations of a variety of living organisms. If the numbers of bacteria in a growing cul-

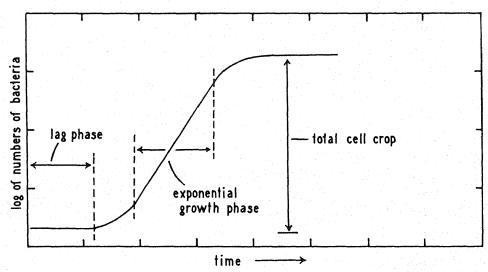


Figure 35. Diagrammatic representation of bacterial growth when the logarithms of the numbers of cells are plotted against incubation time.

ture are measured periodically and plotted against time of incubation, the points fall on a similar S-shaped curve, but one that is asymmetrical in that the point of inflection is not halfway between the upper and lower asymptote. Such curves are ordinarily plotted on semilog paper, i.e., the logarithms of the numbers of bacteria are plotted against time on an arithmetic scale. This procedure gives a more nearly symmetrical curve and has the advantage, not only of minimizing errors in counting, but also, when microorganisms are multiplying at an exponential rate. of the points falling on a straight line, and the generation time being expressed as the slope of the straight-line portion of the curve.

The bacterial growth curve, shown in schematic form in the accompanying figure, is divided into segments, or growth phases. The significant portions are the lag phase during which little or no increase in numbers occurs, the logarithmic or exponential growth rate phase during which the bacteria are multiplying at a maximum rate, and the stationary phase when the maximum total growth has been reached. The positive and negative growth acceleration phases at either end of the period of exponential growth are sometimes differentiated but are of limited utility. Following the stationary phase, death of some portion of the cells present usually occurs, rapidly or slowly and at an exponential rate; microbial death is considered elsewhere (Chap. Six).

It is obvious from even casual consideration of the development of the bacterial culture that descriptions such as "good growth" and "poor growth" have little meaning. Good growth may mean rapid growth and result from a relatively short latent or lag period and/or a short generation time during the exponential growth phase, or it may mean heavy growth in the sense that the maximal population density is high. These are not necessarily synonymous; the cholera vibrio, for example, grows equally rapidly in the logarithmic phase in aerated culture in peptone water, or peptone water enriched by the addition of serum to 10 per cent, but the maximal density is considerably greater in the enriched medium. The meaning of "growth" is, then, variable, and the growth response, however and to what purpose it may be defined, is a function of the environment in the microcosm of the culture vessel.

The lag phase. When bacteria are inoculated into fresh medium an appreciable time, or latent period, is required for adaptation to an environment new in the sense that it differs from that of the established parent culture. There is convincing evidence⁴⁰ that this period is divisible into an apparent and a true lag phase. The first is a consequence of the presence of cells in the inoculum which are not viable in the sense that they will not reproduce, although they may continue to metabolize, and thus reduce the size of the effective inoculum. Within limits, the length of the lag phase is directly related to the size of the effective inoculum, and the numbers of cells present during the subsequent exponential growth phase at successive points in time may be predicted from these considerations.

The true lag phase, in contrast, is the period required for the viable cells of the inoculum to accumulate enzymes, diffusible coenzymes, and essential intermediates in the synthesis of cell substance to the balanced concentrations consistent with synthesis at a maximum rate. When the inoculum is taken from a culture in the exponential growth phase, the lag is largely, or entirely, eliminated since the cells are already in a state of physiological balance making possible synthesis at a maximal rate.

On the other hand, the lag phase may be prolonged indefinitely bvinterference with processes of adaptation. Vigorous aeration, preventing the accumulation of small amounts of carbon dioxide from endogenous respiration that are essential to growth, prevents growth and multiplication. Similarly, the addition of antibacterial substances whose activity is primarily bacteriostatic, i.e., growth-inhibiting rather than killing, results in an indefinite extension of the lag phase. Such substances include those of practical importance as chemotherapeutic agents, such as the sulfonamides and certain of the antibiotics, as well as other substances such as certain dyes. These act by interfering with essential metabolic reactions, such as the synthesis of enzymes (Chap. Six), and when their effect is neutralized, as by p-aminobenzoic acid when growth is inhibited by sulfonamides, growth proceeds. The effect of many chemotherapeutic drugs is, then, in a sense an indefinite prolongation of the lag phase of bacterial growth.

The exponential growth phase. The stage in the development of the bacterial culture during which the cells are replicating at a constant and geometric rate represents an approximate, limited, steady state in the relation between the bacteria and their environment. The steady state is approximate because the environment is continuously altered by the microorganisms, and limited in that it persists over only a portion of the life history of the culture.

The rate of growth is obviously determined by limiting factors. These may be intrinsic in the structure of physiological potentiality of the cell, as, for example, the rate of diffusion across the osmotic barrier of the cell. Or they may be extrinsic in that they may be environmental factors, such as the concentration of an essential nutrient, concentration of oxidizable substrate, and the rate of diffusion of oxygen into the culture.

Continuous flow culture. 48, 58, 59 Bacterial growth in the logarithmic phase may be partially maintained by rapid successive transplant from this growth phase, but much more precisely by cultivation of the bacteria in a continuous flow of fresh medium. A considerable number of devices for this purpose have been constructed of which the chemostat and bactogen have been the most widely used. The former is operated by differential air pressure to provide a continuous supply of fresh medium to a small growth chamber as the used medium exhausted. In the latter the medium is continuously pumped into and out of a large rotating flask in which the bacteria grow in large volumes of culture medium. Both are operated at flow rates held at some fixed value below that required to maintain growth at a maximal rate. The steady state so obtained has been subjected to detailed mathematical analysis. A variation on this theme has been used in which the flow of fresh medium is controlled by a photoelectric system to maintain the numbers (turbidity) of bacteria at a constant level.

Under such constant cultural conditions one factor in the environment, such as concentration of a nutrient substance, may be varied, while the others are held constant in excess so that rate-determining effects may be measured precisely. Further, the kinetics of formation of products of synthesis may be studied in relation to gen-

eration time which becomes an experimental parameter. Theoretically, such a culture could be maintained indefinitely, but in practice it is limited because of the instability of the directive mechanisms of synthesis, *i.e.*, mutations occur and alter the character of the microbial population. On the other hand, the factors affecting spontaneous mutation rates may be studied under constant defined environmental conditions.

Synchronous cell division. 14, 75 In the usual bacterial culture cell division occurs at random to give, because of the large number of discrete events, a continuous increase in numbers of cells. By subjecting the culture to alternate incubation and chilling temperatures, it is possible to force the majority of the cells to divide within a short time and thus synchronize cell division. Studies on pneumococcus and the mouse typhoid bacillus have shown that the entire population is more susceptible to genetic transformation in the first instance, and to conversion to the lysogenic state (see below) in the second. Results such as these suggest that actively dividing microorganisms are relatively more susceptible to alteration of directive genetic mechanisms than are those in a resting state.

Cessation of cell division. Termination of the exponential phase of growth is marked by a relatively rapid decrease in the rate of cell division. Cell division does not stop entirely in the stationary phase, but the cells begin to die and occasional division counteracts deaths so that the number of viable microorganisms remains relatively constant for a time. This period may be relatively short so that within a few hours a decline in numbers of viable cells assumes an exponential rate, or the numbers of viable organisms may fluctuate about some median value that is usually less than the maximum cell density.

The decreasing rate of cell division may be accounted for in many ways, and different factors are operative with different microorganisms, and with the same microorganism under different conditions. Depletion of essential food substances may be the limiting factor but is probably not important with most kinds of culture mediums. The metabolic rate may be reduced by a lessened availability of substrates for the respiratory processes; thus a carbohydrate substrate may be reduced in amount, or

the rate of diffusion of oxygen may be insufficient at high cell densities. One of the most important kinds of factors is the accumulation of end products of metabolism, such as alcohols and organic acids from carbohydrates, to toxic concentrations. For example, the total cell crop obtainable from liquid culture is markedly increased by inclusion of a readily available source of energy, such as glucose, and vigorous aeration. The stirring effect of aeration keeps the substrate concentrations including that of oxygen, more or less uniform throughout the medium and provides excess oxygen so that not only do the oxidations proceed more nearly to completion with reduced accumulation of organic acids and the like, but the energy yield is increased. Under conditions such as these, the limiting factor is glucose concentration, and a cycle of growth may often be started by the addition of more glucose and sufficient alkali to neutralize the small amounts of accumulated organic acids.77

While these energy-yielding reactions and their products are an essential element in that they affect growth, their effect on growth is more precisely defined as that of control of biosynthesis,^{28, 71} expressed as the induction of synthesis of new enzymes as in the early stages of growth, the inhibition of enzymatic activity, and the inhibition of enzyme synthesis. These matters are considered in detail elsewhere (Chap. Five).

Morphological variation. Both the morphology and physiological activity of bacteria differ in the different growth phases of the culture. As growth gets under way, the maximum size that a given cell reaches before fission increases somewhat, and a large portion of the bacteria in a culture at this stage of development are appreciably larger than at other times. The cells stain evenly, and there is no evidence of granular structure even in those bacteria in which metachromatic granules are most readily demonstrable.

The rate of respiration per cell increases, reaching a maximum at the end of the phase of accelerating growth rate, and declining as the culture goes into the logarithmic growth phase. This increased metabolic rate is more apparent than real for it is quantitatively related to cell size.

The cell size decreases also as the culture goes into the logarithmic phase of growth, although the individual cells remain

homogeneous and stain uniformly. By the time the stationary phase is reached, the bacterial cells are uniformly smaller, and in cultures of spore-forming bacteria, many are forming spores. When the viable count begins to decrease, the cells no longer stain uniformly, but begin to show granular structure, and involution forms appear. ¹⁶

This succession of morphological types, the large embryonic or young cells, the smaller, uniformly staining, mature forms of exponential growth, and the senescent degenerating forms appearing in older cultures, has been designated cytomorphosis and is considered by some to be analogous to a similar succession of cell types in higher forms.

THE REPLICATION OF VIRUSES 54, 80, 83, 85, 87

Since the viruses are obligate intracellular parasites, consisting essentially of little more than a directive mechanism, virus replication differs from bacterial multiplication in that it involves a specific deviation in host cell synthetic processes, and the finished product is not a complete physiological system. Virus synthesis can be studied against the base line of normal host cell metabolism in a number of ways, such as the persistence or alteration of metabolic reactions associated with the formation of of virus. Or, the relative proportions of original virus substance, host cell substance, and metabolic substrate present in the extracellular environment that make up the new virus substance may be determined.

The consequences of this parasitic relationship to the host cell are variable. In some instances virus proliferation results in destruction of the host cell, such as the lysis of the bacterial cell by bacteriophage, or the degenerative changes produced in host cells in tissue culture by many of the animal viruses. In other cases, the host cell is relatively unaffected in that it continues an apparently normal existence while supporting virus production, and in still others, viral infection produces hyperplasia. These effects on the host cells in naturally occurring infections are in large part the basis of the pathogenesis and pathology of virus diseases.

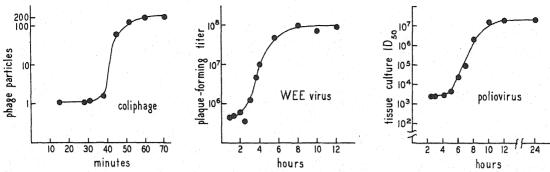


Figure 36. Growth curves of representative viruses. Coliphage and western equine encephalomyelitis virus are shown as one-step growth curves, the latter grown on chicken fibroblast culture and assayed by the plaque-counting method using a monolayer of chicken fibroblasts. The poliovirus type 3 was grown on HeLa cell culture and assayed by tissue culture ID₅₀. (Redrawn from Doermann, Dulbecco, and Ackermann and Francis respectively.)

Characteristics of virus replication. In general, a series of separable steps are involved in the proliferation of viruses. The first is an adsorption, more or less specific, of the infectious agent onto the surface of the host cell. Following adsorption, the particle penetrates the cell, and for an appreciable period, a few minutes to a few hours, cannot be detected as, for example, by breaking up the host cells and assaying the debris for virus activity. This is the latent, eclipse, or dark period during which the virus is said to be in the form of provirus. During this time the synthesis of virus substance is initiated, but active virus is not detectable until the infectious unit is organized through a process of maturation. Prior to this its presence is masked. Such an orderly sequence of events is inferred from the presence of "incomplete" virus in prematurely disrupted host cells, and its occurrence in mixture with complete virus under appropriate experimental conditions. There is no evidence suggesting that anything similar to binary fission of the intact virus particle occurs within the host cell.

In any case, when organization is complete, virus is liberated from the host cell at the end of the reproductive cycle, through breakdown of the cell structure or extrusion from the still functional cell. The liberation may be explosive, as with cell lysis, or virus may be liberated from the cell and shed into the environment over a period. Such a single cycle is called one-step growth. The free virus particles then invade other host cells and/or reinfect cells not destroyed in the previous process, and the cycle is repeated until the host cell substrate is ex-

hausted. In the infected host organism virus replication may be inhibited by the development of an effective immune response and combination of the free virus particles with antibody to interfere with repetition of the reproductive cycle.

In one-step virus growth breakdown of host cells is only partially synchronized in the first cycle, and subsequently becomes random, while continuous liberation of virus from infected host cells results in a continuous increase in virus. In either case, growth curves in which the amount, or titer, of virus is plotted against time are S-shaped and resemble the growth curves of bacterial cultures.

This general pattern of virus replication appears to hold for most of the viruses that have been sufficiently studied, with minor variations. Analogies may be drawn only with reservations, however, because it does not necessarily follow that similar stages in the development of widely different viruses are analogous.

THE REPLICATION OF BACTERIOPHAGE 15, 35

The bacterial viruses have been the most intensively studied of the viruses, largely for technical reasons, and are sometimes regarded as prototypes. While this may not hold true, the pattern of multiplication of these agents includes not only the stages observed with many other viruses, but also elaborations, some of which have not yet been observed with other viruses.

Adsorption.95 The adsorption of the virus particle onto the surface of the bac-

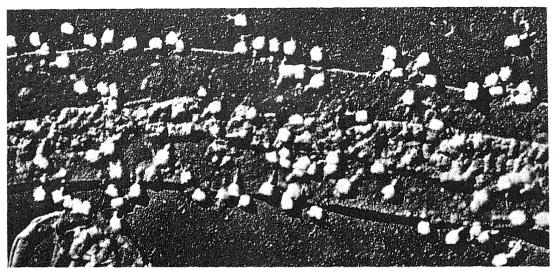


Figure 37. The interaction of T_2 coliphage with cell wall of E. coli B, showing the dissolution of the distal tail protein structure, in some cases leaving the protruding tail spike. The small rod-shaped particles may represent extruded spikes. \times 30,000. (Kellenberger.)

terial cell is highly specific and determines in large part whether or not the bacterium is "susceptible" to infection or, conversely, whether the phage is able to infect the strain or species of bacterium. At least in the Teven coliphages, the portion of the differentiated phage particle structure concerned in adsorption is the terminal portion of the tail, and the phage is adsorbed tail-first on the bacterium. Adsorption is inhibited in the presence of antibody to the distal tail protein which is presumably covered with a layer of immune globulin, but antibody is not effective after adsorption has occurred.

The initial reaction consists of establishing electrostatic bonds between the phage tail and the bacterial cell surface. There is reason to believe that the bonding occurs between amino and carboxyl groups, the

former predominating on the phage particle, and the latter on the bacterial cell wall.⁶⁹ There is some small but by no means complete correlation between the phage sensitivity of a bacterium and its somatic antigens. Phage does combine with purified polysaccharide-lipid somatic antigen, and with the lipocarbohydrate portion of the complex⁴⁹ of certain of the dysentery bacilli. The chemical substrate of the T-even coliphages is a similar substance, assaying 34 per cent lipid and 16.5 per cent reducing substances on hydrolysis, and located in a spherical body deeply embedded in the bacterial cell wall.⁹⁶

Infection of the bacterium.²⁷ For a short time the adsorption of the phage particle on the bacterial cell wall is reversible, but within a few minutes it becomes irreversible

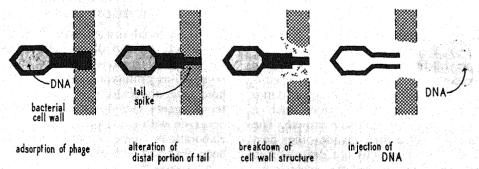


Figure 38. Diagrammatic representation of the sequence of events in the infection of the host cell by coliphage, proceeding from left to right. (Redrawn from Evans.)

when the distal tail protein breaks down, leaving the tail spike intact. As pointed out (Chap. Three), the structure of the distal portion of the tail appears to depend upon the integrity of thiol ester linkages, and there is sufficient zinc in the bacterial cell wall to catalyze the dissolution of the structure.

Following this, the structure of the cell wall breaks down locally with liberation of soluble nitrogen compounds, and the osmotic barrier is affected, allowing some leakage of cell contents. This appears to be associated with contact between the cell wall and the exposed tail structure, resulting in the activation of a heat-labile enzyme which depolymerizes the cell wall structure. Then the tail spike disappears, and the DNA content of the phage head structure is injected into the bacterial cell. The forces responsible for this are not known, but it is possibly relevant that, when the distal portion of the tail is removed by treatment with cadmium cyanide, emptying of DNA is accelerated in the presence of a variety of compounds, such as amino acids, amines, and amino sugars, having in common a positively charged amino nitrogen. On completion of this sequence of events, illustrated in schematic form in the accompanying figure, the attached phage particles may be broken off by treatment of the suspension in a blender. A short portion of the tail remains attached, and the remainder of the particle is an empty head and proximal tail membrane.

These events may also be produced in

vitro in the sense of apart from the intact living bacterial cell. Partial dissolution of the distal tail structure has been referred to above. In addition, phage particles may be adsorbed on purified cell wall preparations or reacted with purified lipocarbohydrate substrate as just described. Soluble nitrogen compounds are liberated in the first instance, and, in both, phage DNA is discharged into the medium with consequent inactivation of the phage.⁵³

Vegetative virus.⁵¹ The term vegetative virus has come to be used to designate proliferating intracellular virus in contrast to the resting stage status of extracellular, nonproliferating virus. The intracellular replication of bacteriophage occurs as a series of distinguishable morphological and biochemical events.

Very soon after infection there is a disruption of the chromatinic body structure, and these bodies are no longer demonstrable as discrete entities. No other overt changes occur until near the completion of the growth cycle when the cell substance has become obviously altered to a filamentous, net-like reticular structure from whose interstices the mature phage particles have escaped.

When the processes of replication are periodically interrupted in various ways, such as mechanical or chemical (cyanide) disruption of the infected cell or inhibition of phage replication by treatment with substances such as proflavine or 5-methyl tryptophan, and the material examined for activity and morphological elements, an orderly process is suggested. During the



Figure 39. The intracellular growth of T_2 coliphage in E, coli B; note that only the polyhedral head portions of incomplete phage are present. Second fixed in osmium tetraoxide. \times 60,000 (Kellenberger.)

early, or eclipse, portion of the latent period, neither phage activity nor distinctive morphological elements are demonstrable. Then spherical, empty bodies, containing little or no DNA, appear in increasing numbers, and these are regarded as phage particle precursors. By the middle of the latent period, mature infectious particles, complete with tails and DNA, may be found. These increase in number, presumably at the expense of the round particles since these decrease concurrently, until the full quota of infective particles, the so-called burst size, is reached, and the cell structure disintegrates to liberate them, leaving behind the reticulated structure described above. The mature phage particle, then, seems to be synthesized piecemeal and then assembled.

The only contribution made by the infecting particle to the new virus substance is a very small amount of DNA, less than 1 per cent of the total new viral DNA, and the remainder of the virus substance is synthesized by the host cell. Consistent with the morphological evidence, there is first an obligatory synthesis of viral protein very early in the latent period. DNA synthesis is occurring seven to eight minutes after the initiation of infection, with the

first mature phage particles appearing 12 to 15 minutes from the beginning of the infection, and continues until all the phage particles are mature and the bacterial cell lyses.

The phage protein is derived in only minor part, 15 to 30 per cent, from the bacterial protein, and is synthesized largely from the constituents of the culture medium. In contrast, practically all of the bacterial DNA is broken down into small fragments and reassembled as phage DNA. When this is insufficient in amount, phage DNA is synthesized from the culture medium. In addition, in the case of the T-even coliphages. the DNA constituent 5-hydroxymethyl cytosine, which does not occur in bacterial DNA, is also synthesized, from bacterial cytosine or from the culture medium. The synthesis of this component would seem to necessitate the formation of enzymes not normally present in the bacterial cell.

lysogeny.^{7,56} Two kinds of bacterial virus may be distinguished on the basis of their relation to the host bacterium. The one, virulent bacteriophage, produces the kind of infection of bacteria just described which culminates in lytic destruction of the host cell, and liberation of mature phage particles. The other kind, temperate bacteriophage, may give the production of

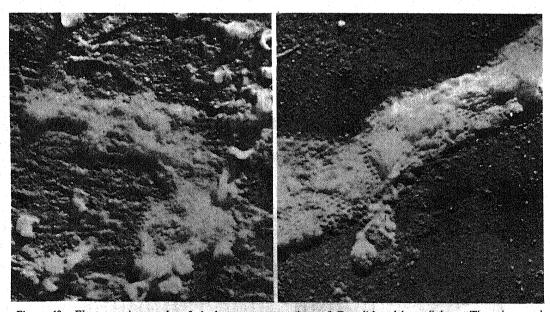


Figure 40. Electron micrographs of shadow-cast preparations of *E. coli* lysed by coliphage. The micrograph on the left shows the filamentous protoplasm; structure can be seen in the filaments just above the central mass, and the structure forms a net in the mass at the bottom. The separate opaque pieces casting long shadows are fragments of bacterial cell membrane. × 15,000. On the right the longitudinal filaments and interweaving cross-filaments are shown in an elongated bacillus undergoing lysis; note the oblique net in the lower left corner. × 22,500. (Wyckoff.)

LYSOGENY 91

active phage and its release by lysis, an abortive infection in which phage is not produced and disappears, or, finally a nonlytic infection in which active phage is not produced but persists in an inactive form indefinitely, i.e., becomes hereditary, in the bacterial host. Treatment of a susceptible strain of bacteria with a temperate phage usually gives some combination of these kinds of infection. A strain of bacteria infected in the last way is said to be lysogenic, and the phenomenon is called lysogeny. Lysogenicity tends to develop more frequently when there is multiple infection of the individual cell, i.e., when the phage is present in excess.

Lysogeny may be mimicked by a kind of carrier state in which virulent phage coexists with the phage-resistant bacteria, multiplying by infection and lysis of phagesensitive mutants occurring occasionally in the bacterial population. The two kinds of association may be differentiated by culture of the bacteria in a medium containing antiphage serum. In the carrier state, or mixed culture, the phage occurs free and is neutralized by antibody so that it is unable to infect the occasional sensitive cell and disappears in serial culture. In the lysogenic strain, the phage persists within the bacterial cells and is transmitted from mother to daughter cell during cell division and so is not affected by serial culture in antibodycontaining mediums.

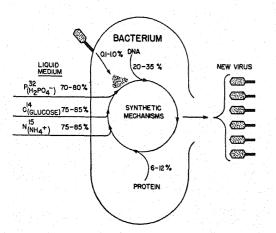
The classic example of lysogenic bacteria is the strain of coliform bacilli, coli-Lisbonne, isolated in 1922 by Lisbonne and

Carrere, which carries a Shiga dysentery bacillus phage. Many stock strains of bacteria are found, on examination, to be lysogenic, and possibly a majority carry phage.

The form in which the virus persists in the lysogenic bacterium is called provirus or prophage, but its nature is not clear. Like virulent phage in the eclipse stage of infection, it is not demonstrable in fragmented lysogenic bacteria, but there is reason to believe that the persistence of prophage represents more than an arrest of the normal processes of phage synthesis and replication. It is generally said that active phage is reduced to prophage, but it is not clear what is meant by reduction.

Virus may be shown to be present in the infected cells by treatment with ultraviolet light, x-ray and other ionizing radiations, nitrogen mustard (methyl bis-[β -chloroethyl]-amine), peroxides, etc. Many of the bacteria are killed since the required dosage is high, but in those which survive, the processes of phage synthesis and assembly are induced. The bacteria are lysed within a short time, perhaps two hours, with liberation of active phage.

The interrelationships between temperate phages and their host bacteria are relatively complex. In general, a lysogenic bacterium cannot be lysed by the active form of the phage it is carrying, and is said to be "immune," a state reminiscent of immunity to superinfection familiar in diseases such as syphilis.^{8, 97} Four others kinds of relationship are observed. First, there may be reciprocal cross-immunity between related



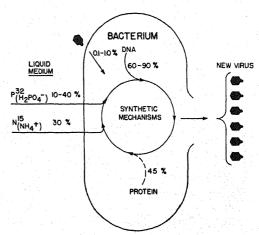


Figure 41. Diagrammatic representation of the source of phosphorus, carbon, and nitrogen in the synthesis of coliphage substance by the host cell. The percentages show the relative amounts contributed to the phage. Left, T-even coliphages; right, T_7 coliphage. (Evans.)

phage strains. Second, there may be a reciprocal sensitivity, i.e., the lysogenic bacterium carrying phage A may be superinfected with phage B, and vice versa. Third, there may be a partial dominance of one phage over another in that the lysogenic strain infected with phage A may be immune to phage B, but the lysogenic strain infected with phage B may be lysed by and/or superinfected with phage A. Finally, there may be complete dominance of one phage over another in that on superinfection the first phage is replaced by the second. In this last relation, the phage strains are said to be incompatible. Given the appropriate relation between phage strains, lysogeny may be multiple, and a lysogenic bacterial strain may carry several phages.

In bacteria infected with virulent phage, the entire directive mechanism of the host cell appears to be replaced by that of the virus, and the physiological system of the cell is literally under new management. In the lysogenic state the directive mechanism of the host cell is supplemented rather than replaced, and the host cell synthesizes provirus in addition to normal cell constituents.

The change may involve the synthesis of still other substances and, as well, minor modifications of the bacterial genetic apparatus. In consequence, characteristics of the bacterium, ordinarily regarded as inherent, biologically fundamental properties of the cell, may be attributable to, or modified by, the presence of phage in the lysogenic state.

These modifications are apparent in a number of ways. For example, the diphtheria bacillus is distinguished from the closely similar diphtheroid bacilli by its production of diphtheria toxin. Toxigenicity, however, has proved to be a characteristic only of lysogenic diphtheria bacilli, and may be removed by abolition of the lysogenic state or conferred on diphtheroid bacilli by making them lysogenic. Furthermore, characteristics of a lysogenic bacterium, either seemingly preexisting, such as biochemical characteristics or content of specific antigens, or acquired, such as drug resistance, may be transferred to another bacterium by making it lysogenic with phage derived from the first bacterium. This phenomenon of transduction, or infective heredity, is discussed elsewhere (Chap. Seven).

THE REPLICATION OF ANIMAL VIRUSES^{12, 61, 73}

Like the bacterial viruses, the animal viruses proliferate only on a living host cell substrate. Those suitable for this purpose are of two general kinds: the embryonated hen's egg and tissue cultures of animal cells such as epithelium and fibroblasts. Some kinds of cells may be maintained indefinitely in transplants like bacterial cultures, while others die off after a few transplants.

In either case, the growth substrate is actively metabolizing host cells which are induced to synthesize virus. The cycle of development is longer than that of the bacterial viruses; complete bacterial virus is formed as early as 20 minutes and seldom later than 90 minutes after the initiation of infection, while the growth cycle of animal viruses is a matter of hours, for example a minimum of four hours for influenza virus and seven hours for herpes simplex virus before the first complete virus is shed from the host cells.

While a living cell substrate is essential in the study of animal viruses, it is useful also in the study of pathogenic bacteria and host cell response. Meningitis may be produced in the chick embryo by the meningococcus; some of the early work with infectious agents in tissue culture concerned the growth of tubercle bacilli in macrophage cultures, and cultured tissue may produce antibody and show hypersensitivity reactions when the explants are taken from immunized animals.

Egg culture. (Chap. Two.) The cells present in the embryonated egg, not only those of the embryo proper but also those of the chorioallantois, the membrane lining the allantoic cavity, and so forth, are susceptible to infection with agents to which the chicken is resistant. This is an expression of the general phenomenon of increase in resistance, or natural immunity, coincident with tissue differentiation. A useful variation is removal of the embryo and culture of viruses in the remaining cells, such as those lining the allantoic cavity, in the de-embryonated egg.

Viral growth may be supported by one tissue but not another, or at one stage of differentiation but not another. For example, rickettsiae are cultivable to large numbers in the yolk sac of the six- to eight-day egg, equine encephalomyelitis virus in the tissues of seven- to nine-day embryos but in the yolk sac of the 15-day egg, influenza virus in the allantoic cavity of the 10- to 11-day egg, and vaccinia virus in the amnionic cavity of the eight- to nine-day egg when the rate of host cell multiplication is sufficient to replace those destroyed, and on the chorioallantois of the 12- to 14-day egg.

The pathology produced may simulate, to a greater or lesser degree, that of the infection in the natural host. The poxviruses, when cultured on the chorioallantoic membrane, produce pocks on the membrane with focal cell proliferation and subsequent necrosis and ulceration, and typical cytoplasmic inclusions. When the embryo is inoculated, tissues of endodermal and mesodermal origin are infected, and pock-like eruptions occur on the skin. Similarly, the virus of herpes simplex, when inoculated on the chorioallantois produces necrosis of ectodermal epithelium, and then infects mesodermal cells, and inclusions are found in endothelial cells. A herpetic encephalitis is produced in the embryo inoculated by the intracerebral route, and inoculation of the amnionic cavity results in vesicular lesions of the skin and mucous membranes of the pharynx in addition to a general infection of amnionic cells. On the other hand, cultivation of rickettsiae in the yolk sac, or influenza virus in the allantoic cavity, does not result in pathology similar to the natural infection or that induced in experimental animals.

Tissue culture. 82 Cultured cells for virus propagation may be primary explants, as in the case of monkey kidney cells, or established cell lines which may be carried indefinitely, like bacterial cultures, and are known by various designations such as L cells. HeLa cells, and KB cells. The cultures may be as monolayers on which some viruses produce grossly apparent discrete areas of cell pathology, known as plaques, and which are analogous to bacterial colonies. So-called spinner cultures, in which the cells are suspended, are useful also. The elements of basic cell culture techniques are described elsewhere (Chap. Two), and in considerable detail by others.70

Cytopathogenesis.²⁶ The proliferation of viruses is commonly, though not invari-

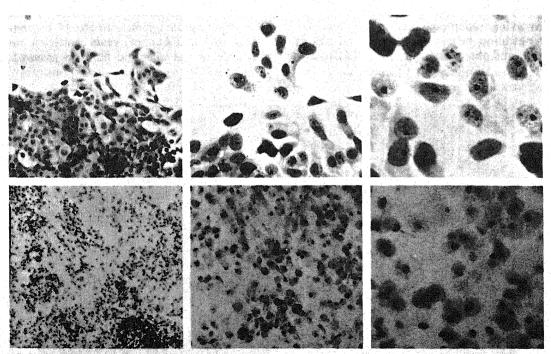


Figure 42. The cytopathology produced in HeLa cell culture of poliovirus type. I. Top row, uninfected tissue shown at magnifications of \times 65, \times 160, and \times 250, reading from left to right. Bottom row, degenerative changes produced in two days shown at the same magnifications reading from left to right. (Scherer, Syverton, and Gey.)

ably, accompanied by changes in the host cells, the cytopathogenic effect of viruses. The kinds of visible changes produced allow the separation of viruses into groups, viz., those that produce only cellular degeneration, those that produce degeneration and also form inclusion bodies in the degenerated cells, and those that produce multinucleated cells or multinucleated syncytial masses of protoplasm together with degenerative changes with or without the formation of inclusions.

The first group includes agents such as the polioviruses, the Coxsackie viruses, foot-and-mouth disease virus, and Newcastle disease virus (NDV); the second includes poxviruses such as mouse pox (ectromelia) and vaccinia, fowl plague virus, and the viruses of the psittacosis-lymphogranuloma group; and the third group includes viruses such as those of herpes simplex and herpes zoster and varicella. A fourth group of viruses which grow in tissue culture without apparent visible changes in the host cells includes the viruses of influenza, St. Louis encephalitis, and yellow fever.

Developmental stages. The replication of animal viruses in egg or cell culture follows the general pattern described above. The initial absorption is specific, presumably involving receptor sites on the host cell. Penetration begins very soon after absorption, and the animal viruses differ from the

bacterial viruses in this respect, in that the process involves pinocytosis, with the cell playing an active role.^{21, 67}

While there may be some alteration of the virus particle on attachment, the viral nucleic acid is released from the nucleocapsid only after the particle has entered the cell. Following the eclipse period,⁵⁰ mature virus is released more or less continuously, and successive replication cycles may occur within the same cell, since the cell is not destroyed after a single cycle as in the replication of phage. In general, the entire process is much slower than that of bacterial virus replication, and usually takes several hours.

The animal viruses differ from one another in the details of the replication process; in some cases, for example, the mature virus particle is formed within the host cell, and in others assembly seems to be completed at the periphery of the cell and just prior to release. Influenza virus, herpesvirus, poliovirus, and poxvirus are considered here for illustrative purposes.

Influenza virus.³⁷ Proliferation of influenza virus in the entodermal cells lining the allantoic cavity of the embryonated hen's egg occurs readily, and it is one of the best known among the animal viruses in this respect. Following inoculation into the allantoic cavity of a 10- to 11-day embryo, the number of virus particles detectable in the allantoic fluid decreases at

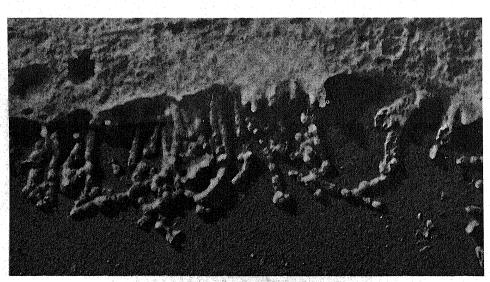


Figure 43. Shadow-cast ultrathin section of chorioallantoic membrane infected with influenza virus. The filaments extruded from the cells of the membrane appear to abstrict into spherical particles. × 10,000. (Wyckoff.)

an exponential rate, about 50 per cent disappearing in a little over an hour, as the virus is adsorbed to the host cells.45 During this early stage, infection of the cells may be prevented by RDE, i.e., neuraminidase, which destroys the red cell receptor functional in influenza virus hemagglutination (Chap. Three), and there is reason to believe that the host cell and red cell receptors are similar. Following adsorption, the virus particles enter the host cells, and the eclipse period lasts about four hours.

The first evidence of viral multiplication is the appearance in the middle of the eclipse period of soluble antigen, detectable by the complement-fixation test, which is not infective and has no hemagglutinin activity. In this connection it is of interest that, when the mature virus particle is disintegrated by shaking with ether, it breaks down to soluble complement-fixing antigen and hemagglutinin, suggesting that the soluble antigen may reflect an intermediate stage in virus synthesis.

Hemagglutinating activity, but not infectivity, is found in allantoic membrane extracts three to four hours after inoculation but does not increase in amount over that originally present in the inoculum until four hours and later. The hemagglutinating activity is not infective, and infective virus appears five to six hours after inoculation and is shed almost immediately into the allantoic fluid.23

Morphological studies of ultrathin sections of allantoic membrane by electron microscopy at this time show both spherical mature virus particles and filamentous structures, but no characteristic changes in the nuclei or cytoplasm of the host cell. The filaments appear to be formed by extrusion from the cell wall¹⁷ and are similar to those that may be produced by changes in osmotic balance. Both filaments and spheres appear to consist of a limiting membrane, a diffuse layer, and the spheres contain a dense central body about 20 m μ in diameter. The spheres seem to differentiate at or just beneath the cell surface, with assembly of the internal body, external layer, and limiting membrane.64

The relation between the filaments and spheres is not entirely clear. It is believed by some that they are separate structures, but others believe that the filaments are, or differentiate into, rows of spheres. The filament is broken down by treatment with acid, and the spheres are digested by trypsin after acid treatment.88

However this may be, the spherical forms, 60 to 70 mµ in diameter, represent the mature, hemagglutinating, infectious form of the virus. About 40 to 60 such particles are liberated in one growth cycle. The cycle is repeated by infection and reinfection of the cells lining the allantoic cavity, with the accumulation of virus in the allantoic fluid, until the system is exhausted after about 48 hours.

INCOMPLETE VIRUS.5, 57 Under appropriate circumstances incomplete, i.e., functionally deficient in that it is noninfectious, influenza virus may be produced. Deficient virus particles occur when the allantoic cavity of the embryonated egg is inoculated with very large amounts of virus, when the virus is grown in the allantoic cavity of deembryonated eggs, or when nonneurotropic (nonadapted) strains of the virus are grown in the mouse brain.

In the first instance, the inoculum must be sufficiently heavy, more than 1 hemagglutinating unit (HU) of virus, to give multiple infections of the allantoic cells; there are about 1×10^8 cells lining the allantoic cavity, and 1 HU of virus contains 1×10^6 to 1×10^7 virus particles. Presumably the virus-synthesizing capacity of the allantoic cell is limited, and when this limit is exceeded the cell responds by incomplete synthesis of a portion of the virus formed. In the second instance, removal of the embryo seems to affect the allantoic cells in such a way that they can support only partial synthesis of virus. In the third, virus has not been adapted to the host cell substrate present in the central nervous system in the sense that these cells are not fully competent to synthesize it.

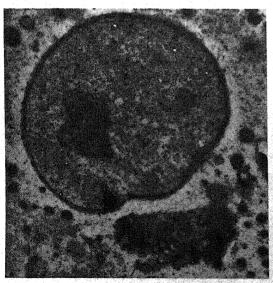
Incomplete or deficient virus differs from mature virus in that, while it agglutinates red blood cells and is specifically adsorbed to them, it is not only not infective and therefore unable to proliferate, but also it is smaller, with a sedimentation constant of 500 S as compared with one of 750 S for mature complete virus. A variety of considerations make it clear that incomplete virus is not a degradation product, but its appearance represents an arrest of the processes of synthesis and assembly of the mature virus particle.

Herpesvirus. 65, 78, 81, 93 Studies on the growth of other animal viruses have shown that the same general sequence of events, viz., adsorption, eclipse, and assembly and shedding of the virus, occurs with minor variations. Herpes simplex virus is of interest because of the formation of excess DNA within the nuclei of infected cells and the intranuclear formation of the virus. The virus is adsorbed rapidly on the chorioallantoic membrane of the embryonated egg. 50 to 80 per cent of the virus particles being adsorbed on the ectodermal cells in 15 minutes, and the adsorption is substantially complete in an hour. Adsorption is slower, e.g., 50 per cent in one hour, and two hours are required for complete adsorption in HeLa cell culture. 43, 94 The latent period is six to 12 hours, and virus increases exponentially for 10 to 20 hours thereafter, but excess DNA is demonstrable in the nuclei of infected cells as early as six hours after infection.89 The virus particles are demonstrable within the nuclei as nucleic acid enclosed in a single-layered protein coat. A second protein coat is acquired elsewhere. presumably in the cytoplasm, prior to shedding of the virus.

Poliovirus.^{22, 44} Poliovirus grown in monkey kidney and other cell cultures is completely adsorbed under optimal condi-

tions in two hours, and the eclipse period persists for three to four hours. New virus begins to accumulate intracellularly, and it has been reported that protein subunits. which appear in the cytoplasm, begin to aggregate as early as three hours after infection, leading to subsequent appearance of the typical icosahedral virus particle. There is evidence that protein and RNA³⁸ are synthesized independently, but not that RNA synthesis occurs in the nucleus. Recombination experiments have suggested that the protein aggregation about viral RNA tends to be indiscriminate, but in general the processes of synthesis of viral substance and maturation of the vegetative particle are poorly understood. Complete virus begins to be shed as early as five hours after infection, apparently relatively rapidly by the individual cell, but shedding is asynchronous and continues in the culture for 18 to 24 hours after infection.²⁴ The infected cells show marked morphological changes in nucleus and cytoplasm, tending to disintegrate as the virus is shed, and the cell is killed in a single cycle of virus replication.3

Poxviruses. 13, 55, 62, 79 Viruses of the pox group have been studied in some detail in this connection, and the observed morphological types of vegetative virus suggest an orderly sequence in the forms occurring



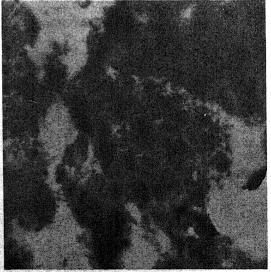


Figure 44. Electron micrographs of the developmental bodies of vaccinia virus. Left, early infection of a chorioallantoic cell of the embryonated egg, showing the nucleus of the cell and below it the matrix from which the virus particles originate. The developmental bodies in the matrix are few and appear to be "empty" membranes, and electron-dense mature virus particles are scattered in the cytoplasm. × 4000. Right, the differentiation of virus in an inclusion body in a rabbit corneal cell, showing the transformation of a part of the inclusion into virus particles, and developmental bodies of the virus in various stages of maturation. × 16,500. (Gaylord and Melnick.)

during the process of replication. Unlike influenza virus, which seems to be assembled in final form at or near the cell surface and shed almost immediately into the environment, at least some of the poxviruses are formed in masses of differentiated protoplasm well within the cell, and the cytoplasm of the host cell may be filled with mature virus particles in the later stages of infection. When the masses of differentiated protoplasm are dense, they constitute the typical inclusion body, and when they are less dense, i.e., inclusion bodies do not occur, the differentiated protoplasm constitutes a matrix from which the virus particles develop. This process has been studied in detail by Gaylord and Melnick²⁹ by electron microscopy of ultrathin sections of infected tissue embedded in methylacrylate, using vaccinia, ectromelia (mouse pox) and molluscum contagiosum viruses. These viruses are similar with respect to the morphology of both developmental and mature forms.

During active multiplication several kinds of particles may be seen. These include hollow spheres, spheres filled with material of low electron density, spherical-to-ovoid forms in which there is a small round, or larger bar- or dumbbell-shaped body of high electron density and finally ovoid bodies

of uniform high electron density that represent mature virus. Since the hollow spheres predominate early in the infection, and those containing electron-dense material predominate late in the infection, it may be inferred that the several morphological types of particles arise from one another in orderly sequence, the hollow spheres representing an early stage in the developmental cycle. The process appears to be the same whether the virus is formed in an inclusion body or in a matrix, though possibly less confined in the latter instance. For example, the same developmental forms of vaccinia virus are found in the cells of the chorioallantoic membrane of the embryonated hen's eggs where inclusion bodies are not common and in the rabbit corneal cell where they are produced regularly.

Infectious nucleic acid.^{20, 39} It is apparent from the nature of the replication cycle of bacterial viruses that the entry of viral nucleic acid into the host cell is the essential feature which determines the synthesis of virus by the host. A corresponding function of RNA of many animal viruses is directly demonstrated by the preparation of RNA from the virus as infectious nucleic acid which will similarly infect the host cell and induce it to form, not only the nucleic acid, but also the complete vegetative virus

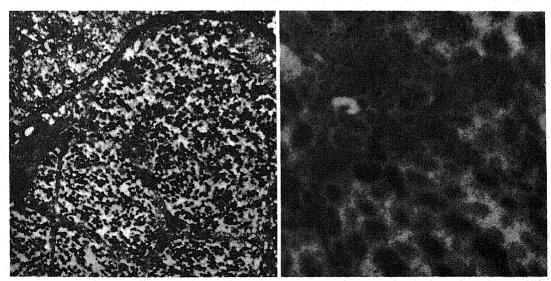


Figure 45. Electron micrographs of molluscum contagiosum virus in naturally infected cells. Left, a cell packed with mature virus particles separated into pockets by strands of cytoplasmic material. The nucleus is pushed to one side, and the nucleus is very dense. × 8000. Right, virus particles under high magnification, showing all the developmental stages from the virus precursors clustered in the remnant of cytoplasm to those having bar- or dumbbell-shaped masses of dense material in the center, and the mature uniformly dense particles × 26,000. (Gaylord and Melnick.)

particle. Extraction of RNA in 50 per cent phenol was described by Gierer and Schramm³⁰ for tobacco mosaic virus, and has subsequently been applied to a variety of RNA-containing animal viruses, for the most part small, neurotropic viruses, including poliovirus, the equine encephalomyelitis viruses, and certain of the arthropod-borne, ECHO, and Coxsackie viruses. The infectivity of such preparations is demonstrable by the inoculation of intact animals, the embryonated egg, and various kinds of tissue cultures, and is destroyed by treatment with ribonuclease. The yield of infectivity is low, commonly on the order of 0.1 per cent of the virus preparation from which the nucleic acid is extracted, but may be improved by use of hypertonic, on the order of 1 M., saline or sucrose solutions; such hypertonic solutions apparently both stabilize the nucleic acid infectivity and facilitate its entrance into the host cells.

Certain observations with soluble preparations of DNA derived from bacterial virus may be analogous. Solutions of the virus substance obtained by osmotic shock lysis and by extraction in concentrated urea infect host cell protoplasts which are not infected by intact virus. The activity is presumably that of DNA, especially in the urea-treated preparations, but the presence of functional protein is not completely excluded.

Host cells and tissues. 18, 31, 52, 72, 84, 91 As pointed out earlier, the synthesis of virus by a host cell represents a deviation of the normal processes of metabolism through the imposition of a new directive mechanism. The characterization of the processes of virus synthesis against the base line of normal host synthesis is one of both theoretical and practical significance. 34, 42

In general, virus proliferation appears to be an obligate aerobic process, occurring most actively, and usually exclusively, in the actively metabolizing host cell. There is little overt evidence of infection, viz., the endogenous respiration rate is not altered by infection. When the tissue is glucose-starved, virus production is enhanced by the addition of glucose and, to a lesser extent, pyruvate or alanine. Intact functioning of the citric acid cycle is apparently essential, and virus production is inhibited by substances such as fluoroacetate.

It is of considerable interest that the viral

infection may persist in host cell cultures which continue to multiply, resulting in a "carrier" state. 63 Walker 92 has distinguished four kinds of carrier culture: (a) those in which the majority of the host cells are genetically resistant and the virus infection persists in susceptible cells, which are in a minority: (b) those in which the host cells are susceptible to infection, but antiviral factors in the medium limit transfer of virus from cell to cell; (c) those in which the host cells are susceptible to viral infection, but interfering substances produced in the cell culture make the majority temporarily refractory; and (d) those in which the host cells are infected but not destroyed, the infection persisting through cell division. Such cell-virus relationships are not mutually exclusive, and more than one kind may occur simultaneously and/or a succession of such relationships may occur.

Cytotropism. There is a considerable degree of specificity between a virus and the kind of cell or tissue in which it is able to grow, i.e., which provides an appropriate substrate. It has already been noted in connection with virus culture in the embryonated egg that some viruses will grow in some tissues but not in others. The combination of tissue differentiation in higher animals and the obligate intracellular growth of viruses provides the basis for tissue predilection or cytotropism of viruses. This is not a unique feature of these agents for pathogenic bacteria may also show a similar affinity for certain tissues such as the preferential infection of the central nervous system by meningococcus, the predilection of typhoid and related bacilli for the bone marrow, and the relative noninfectivity of the cholera vibrio on parenteral inoculation, which is known as localization or elective localization rather than cytotropism.

Viruses are separable into a number of groups on this basis, that is, neurotropic, dermotropic, pneumotropic, and viscerotropic viruses. The neurotropic group includes agents such as the encephalitis viruses, which produce disease primarily of the central nervous system; the dermotropic group is exemplified by the poxviruses; the viscerotropic group is typified by yellow fever virus; and the pneumotropic group includes influenza, pneumonitis, and similar viruses.

Cytotropism is accentuated among the

viruses, fundamentally because of their complete dependence upon host cells for proliferation, and superficially because the symptoms of the diseases they produce derive in large part from the pathology of some kinds of tissues to the relative exclusion of others. It is, however, regarded as of less importance than formerly because it may be altered, and because virus-infected tissues may show no pathology and not contribute to disease symptoms. In the first instance, for example, naturally occurring viscerotropic yellow fever virus, dermotropic vaccinia virus, and pneumotropic influenza virus may be made neurotropic by passage in the mouse brain, etc. In the second place, it has become increasingly clear that the pathogenesis of at least some virus diseases involves multiplication in tissues other than those associated with the symptoms of the disease; poliovirus, for example, apparently multiplies in the tissues of the alimentary tract without histopathology and reaches the central nervous system by hematogenous spread.

Cytotropism remains a fundamental characteristic of viruses in that it is a consequence of the ability of the virus to multiply more readily in one kind of cell than in another, but it is a characteristic that is

subject to change.

MULTIPLE INFECTIONS AND INTERFERENCE

In view of the obligate intracellular multiplication of viruses, the interesting question of whether or not multiple infections may occur presents itself. While the undoubtedly complex interrelationships of viruses with one another and with the host cell are not yet fully understood, it is clear that multiple, often double infections may or may not occur, depending upon the viruses involved and upon the circumstances of infection.

Multiple infections. Two kinds of multiple infections may occur. The one, multiple infection of an animal, is spurious for present purposes when different cells and tissues are involved. The other kind of multiple infection is that in which the same host cell is infected simultaneously with more than one kind of virus, supporting independent growth of each, and at the same time continuing, to some degree, its own me-

tabolic activities. One such multiple infection, that of lysogenic bacteria with temperate bacterial viruses, has already been described. Among the other viruses, this may occur in some combinations of viruses, but not in others. One of the best examples is the simultaneous infection of the rabbit corneal cell with vaccinia and herpes simplex viruses in which the characteristic intranuclear herpetic inclusion and the cytoplasmic inclusion body of vaccinia may be found in the same cell. When the viruses are unrelated, such as these, the infections develop independently in that the viruses do not affect one another.

When a multiple infection with closely related viruses, such as the T-even coliphages or influenza virus variants, is produced by simultaneous exposure of the host cell, during the course of the mixed infection which occurs, there may be interaction between the two viruses, with recombination of characteristics and the appearance of

hybrid progeny (Chap. Seven).

Interference. 46, 90 Interference viruses occurs when a second infection cannot be superimposed upon an existing infection, or when one virus infects, i.e., multiplies, to the exclusion of a second. In the first instance it is necessary to establish infection with the first virus by prior inoculation, allowing a period at least half as long as that required for a single growth cycle to elapse before infecting with the second. For example, while mixed infections with T-even coliphages may be produced by simultaneous inoculation as indicated above, if infection of the bacterium with one phage is first established, the cell cannot be infected with the other phage, and the same holds true for influenza virus strains inoculated into the allantoic cavity of the embryonated egg. Similarly, mumps and influenza viruses are incompatible in that infection with one cannot be superimposed on an existing infection with the other.

In the second instance, when the interfering virus is inoculated in very large amounts it markedly inhibits growth of the other virus, resulting in either apparently complete protection or an abortive infection. For example, the intracerebral inoculation of nonadapted influenza virus in mice, incapable of more than a single multiplication cycle in this tissue, gives a high degree of protection against virulent western equine

encephalomyelitis virus by the same route for as long as a week, and normal susceptibility is not reached for three weeks. Similarly, under different experimental conditions, infection of rats with the same virus by the intranasal route is inhibited by St. Louis encephalitis or Japanese B encephalitis viruses given by the same route, with a reduction in mortality from 100 per cent to about 5 per cent. The latter kind of experiment illustrates the fact that interference is probably seldom fully effective, and some multiplication of the second virus may occur. It is not known whether this is due to a few host cells, which escaped the first inoculation, being infected by the second, or whether multiple infection occurs in some small number of cells.

Interference is not related to the initial adsorption stage of infection, nor is it produced by soluble components of viruses, but is apparently a consequence of the relation between the virus particle and the host cell after the synthetic mechanisms of the latter are affected.

Kinds of interference. The interference relationships between viruses are of two general kinds. The interference may be reciprocal in that either of a pair of viruses will prevent superinfection by the other. Or the interference may be effective in only one direction, i.e., one virus may be unable to superinfect, but the other can infect in the presence of an infection with the former. A third kind of relationship is, of course, that of noninterference resulting in the development of multiple infections of the kind described earlier. There appears to be little correlation between the kinds of interference effects, or lack of them, and other characteristics of the viruses concerned, and in this sense interference is nonspecific.

AUTO-INTERFERENCE. Attenuated or inactivated viruses may produce interference effects in heterologous systems, i.e., against diverse viruses, and they also inhibit multiplication of the corresponding homologous active virus. It was noted above that mixed infection of the allantoic cells of the embryonated egg may occur when the allantoic cavity is inoculated with variant strains of influenza virus. As in the case of the T-even coliphages, when infection with one strain is established, infection with the second does not occur. The "immunity" of lysogenic bacteria to lysis with the same

phage in active form is possibly analogous, with provirus interfering with active virus synthesis. Protection of the fox against infection with virulent distemper virus by the inoculation, either simultaneously or during the early incubation period, with large amounts of the ferret-adapted virus is a similar interference phenomenon.

When inactivated virus is used to interfere, the way in which it is inactivated is of some importance. Inactivation of influenza virus by treatment with formal-dehyde destroys the interfering property, inactivation by heat gives variable results, and inactivation by ultraviolet radiation gives uniformly effective interfering preparations. In the case of influenza virus, incomplete virus also interferes with subsequent infection with active complete virus.

MASKED VIRUS. Auto-interference also occurs when mixtures of inactive and active virus are used to produce infection, provided that the former is present in great excess, i.e., a thousand-fold or more, and in sufficiently high concentration. Mixtures of active and inactive virus may be produced by actual mixing, by partial inactivation, intentional or unintentional, or when incomplete virus is formed in relatively large amount. When such a mixture is inoculated in cell culture or susceptible animals, there is little or no evidence of the presence of active virus because of the interfering effect of inactive virus, and the viral activity is said to be masked.

Such a masking effect may occur when seed virus is stored for a long period or under conditions that cause the greater part of it to be inactivated. Similarly, this effect makes it difficult to titrate the end point of virus destruction with confidence, but in either case simple dilution of the inoculum may allow the demonstration of active virus if there is still sufficient present to allow the necessary dilution.

In animals infected with certain viruses, especially some tumor viruses, the activity may tend to disappear in serial animal passage by transplant of infected tissue, and the activity is said to be masked. It is possible that such masking may be due in part to the presence of inactive virus, but other factors, such as the concurrent presence of antibody, are significant also.

Nature of interference. It seems clear that viral interference needs to be considered as occurring at the cellular level. One

of the more attractive theories of interference is that of a competitive inhibition of incompatible viral nucleic acids, but this is not completely consistent with the interfering effect of inactivated virus.

Interferon.^{4, 11, 32} The resistance of cells to virus infection following pretreatment, or simultaneous inoculation, with inactivated or avirulent homologous or heterologous virus has been found to be attributable, at least in many instances, to the production by the host cell of substances known collectively as interferon. This phenomenon was first observed with myxoviruses, but it has been found subsequently that interferon production is stimulated by many viruses.

Interferon has been of very considerable interest in connection with its possible role in resistance to, and recovery from, viral infections. Functionally, its inhibitory activity is demonstrable as transfer of resistance from one cell culture to another in the absence of the initially interfering virus. It is quite specific with respect to species of host cells but has a relatively broad spectrum of antiviral activity, the arboviruses being the most resistant and the myxoviruses the most sensitive to it. It has been prepared in purified form, and appears to be a protein, is trypsin-sensitive, and has a molecular weight of 25,000 to 30,000.

It has no antiviral effect on extracellular virus, nor does it combine with viral nucleic acid, and apparently requires the mediation of the host cell. Pretreatment of cells with interferon does not result in immediate resistance to challenge infection, but an incubation period of several hours is required before maximal resistance is reached. It apparently stimulates the cells metabolically, in that aerobic glycolysis is increased concurrently with a decrease in phosphate uptake and utilization of glucose via the pentose shunt. Such evidence has been taken to imply that interferon itself may have no antiviral activity but rather serves to mediate such activity. In any case, it seems clear that the antiviral effect occurs in an early stage of virus replication, following uncoating of the virus particle, with prevention of the synthesis of viral nucleic acid.

The formation of interferon is a property of actively metabolizing cells, and that it is largely synthesized in response to an inducer is indicated by inhibition of its formation by actinomycin-D, an antibiotic which inhibits DNA-controlled RNA synthesis. While it appears to be largely newlyformed in virus infections, some may preexist as indicated by its appearance in the blood in response to endotoxin inoculation. It is probable that the capacity to produce interferon is inherent in the cells, but is ordinarily repressed in the absence of an inducer.

THE COMPARATIVE PHYSIOLOGY OF GROWTH

The physiological capabilities of microorganisms are an inverse reflection of their growth requirements in that the synthesis of cell substance from inorganic and simple organic compounds is a more complex process than that from more direct precursors of the constituents of protoplasm. Taken as a whole, microorganisms range in continuous series from the autotrophic forms capable of living in an inorganic environment, deriving energy from the oxidation of inorganic substrates or by a photosynthetic process. and assimilating nitrogen, carbon, etc., from inorganic sources, to the obligate intracellular parasites incapable of any, or at best rudimentary, independent physiological activity. The details of the processes of respiration, synthesis, and assimilation are considered elsewhere (Chap. Five).

In the strict sense of the term, only autotrophic microorganisms are not parasitic in that they are not dependent to some degree on other living organisms. Physiological limitations begin to appear in the heterotrophic forms, whose characteristic requirements of organic substrates for growth appear first as a requirement for organic carbon both as a source of energy derived by oxidation and as a source of intermediates for the synthesis of cell substance. Among the more fastidious kinds of microorganisms. the requirements for growth become more specific in that certain molecular structures. such as those represented in amino acids and fragments of enzymes or enzyme precursors, must be supplied preformed, but the microorganism is still physiologically independent in that it constitutes a complete physiological system.

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Finally, the obligate intracellular parasites are set apart in that they are not complete physiological systems but are de-

pendent upon the synthetic mechanisms of living host cells for their replication. It is more than coincidence that lack of independent metabolic activity is associated with the most minute forms, for there is literally not room within the small virus particle for the unknown but relatively large number of enzyme molecules required by a physiologically independent unit.

Since growth is the replication of the individual unit, and the replication is necessarily a directed process if individuality is maintained, microorganisms of diverse physiological capabilities may be equated on this common ground. The directive mechanism, or genetic control of synthesis, lies in pentose nucleic acid compounds, the deoxy form, or DNA, in bacteria and some viruses, and the RNA type in other viruses.

The mechanisms of the replication of DNA in situ by DNA polymerases and the direction of the processes of protein synthesis through the mediation of the various kinds of RNA and ribosomes (Chap. Three) have by now become clarified. In the bacteria the mechanisms of respiration and synthesis of the complete organism are present in each cell, but the viruses contain only the directive apparatus, conventionally enclosed in the capsid but active in the free state as infectious nucleic acid, and they parasitize the host cell with respect to high energy-yielding reactions and the synthetic functions of ribosomes (Chap. 3). Differences among microorganisms would appear. then, to be those of degree rather than kind.

The growth of microorganisms can be regarded primarily as an expression of pentose nucleic acid replication, complicated to a greater or lesser extent by the side reactions of synthesis of the remainder of the unit. Such a view not only places the multiplication of diverse microorganisms on a common ground, but also provides a basic working hypothesis for an interpretation of the ways in which the hereditary mechanisms of these forms may be altered (Chap. Seven). 83

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Chapter Five

BACTERIAL METABOLISM

By JAMES W. MOULDER, Ph.D.

One of the fundamental principles of biology is that the chemical composition of all organisms is essentially the same and that their cell substance is synthesized by the same kind of chemical reactions; that is, the metabolism of all living things fits a single general pattern, a reflection of a common evolutionary origin. The complex edifice of cell structure is built from a relative handful of simple building stones needed for the life of all cells. These essential metabolites are synthesized by the same mechanisms and enter into the same reactions in organisms of diverse phylogenetic origin.

However, this concept of the unity of biochemical processes does not mean that the composition and metabolism of all cells is exactly the same. In fact, the difference between bacterial metabolism and the metabolism of higher organisms is the subject of much of this chapter. As already discussed, bacteria have many morphologic characters, such as the absence of a nuclear membrane, which show that they are primitive cells residing on branches near the base of the phylogenetic tree. Bacteria are also biochemically primitive, but in the sense of being unspecialized, not of being simple. It appears as if primordial bacteria often evolved a number of different ways of performing a specific metabolic task but that only one was selected for use in higher cells. However, these diverse metabolic mechanisms frequently persisted in bacterial evolution and may be found in present-day bacteria. For example, the only way animal cells can generate metabolic energy is by oxidizing one of a small number of different organic compounds. On the other hand, among present-day bacteria, there are those that can get metabolic energy from light, from oxidation of inorganic compounds, and from oxidation of a wide variety of organic compounds.

There is another fundamental difference between the metabolism of bacteria and the metabolism of higher plants and animals. As part of a multicellular organism, the plant or animal cell lives in a uniform internal environment and does not have to shift its metabolic patterns to adapt to sudden changes in its immediate surroundings. In contrast, the bacterial cell lives in the midst of an environment that changes frequently and violently. It copes with these changes by changing itself. A typical bacterium carries in its genome the information for making many more enzymes than it ever needs at any one time. In general, it will synthesize only those enzymes it needs for multiplying in a specific environment, and it will frequently cease this function as soon as an adequate amount of the products of this activity have been synthesized. Thus bacteria have a far greater metabolic flexibility than do higher cells, and under different cultural conditions a given bacterium may exhibit a diversity of metabolic pathways.

Respiration

In its broadest sense, bacterial respiration refers to all the energy-yielding reactions occurring within the cell. Except in photosynthetic organisms, these reactions consist of the oxidation of chemical substances present in the medium in which the bacteria are growing. Respiration has two main and often inseparable functions: (1) it furnishes the energy needed for the life and growth of bacteria and (2) it transforms a considerable portion of the compounds oxidized as energy sources into substances which are assimilated and synthesized into new cell material.

SOME BASIC CONCEPTS OF RESPIRATION

When one substance is oxidized, another must simultaneously be reduced. The oxidized substance loses electrons which are gained by the one reduced. This transfer of electrons is easily seen in the oxidation-reduction of inorganic ions. In the example given here, Fe⁺⁺⁺ clearly accepts an electron from Cu⁺ and is thus reduced to Fe⁺⁺.

$$Cu^+ + Fe^{+++} \rightleftharpoons Cu^{++} + Fe^{++}$$

When organic compounds are oxidized, as in most reactions of bacterial respiration, hydrogen atoms are transferred as well as electrons. Thus, malic acid transfers two hydrogen atoms and two electrons to pyruvic acid with the result that malic acid is oxidized and pyruvic acid is reduced, as shown in 1.

The majority of biological oxidations may be most simply considered in terms of hydrogen transfer, although it must be remembered that electrons are transferred in the oxidation of organic compounds, just as in the oxidation of inorganic ions.

Using the concept of hydrogen transfer, any biological oxidation may be considered to be a special case of this general reaction.

$$A-H_0+B \rightleftharpoons A + B-H_0$$

A-H₂ is the substance oxidized or the hydrogen donor, while B is the substance reduced or the hydrogen acceptor. One other component of the system is still needed. The oxidation-reductions of respiration do not proceed spontaneously, but require the presence of specific cell catalysts, the respiratory enzymes.

Biological oxidations are usually classified as either *aerobic* or *anaerobic*. If the hydrogen acceptor is molecular oxygen, *i.e.*,

$$A-H_2 + \frac{1}{2}O_2 \rightarrow A + H_2O$$

the oxidation is said to be aerobic. If a

O OH OH OH CH₃—CH₃—CH₂—COOH
$$\rightleftharpoons$$
 CH₃—CH—COOH \rightleftharpoons CH₂—COOH \rightleftharpoons CH₃—CH—COOH \rightleftharpoons CH₂—COOH \rightleftharpoons Dyruvic acid malic acid oxalacetic acid

2.

Some Typical Energy-Yielding Reactions in Bacteria

ORGANISM
Nitrosomonas europaea
Thiobacillus thiooxidans
Thiobacillus denitrificans
Hydrogenomonas pantotropha
Methanobacterium omelianskii
Escherichia coli

Lactobacillus casei

Clostridium kluyveri

Clostridium sporogenes

REACTION NH₃ + 1½ O₂ \rightarrow HNO₂ + H₂O S + 1½ O₂ + H₂O \rightarrow H₂SO₄ 5 S + 6 KNO₃ + 2 H₂O \rightarrow K₂SO₄ + 4 KHSO₄ + 3 N₂ H₂ + ½ O₂ \rightarrow H₂O \rightarrow C₄H₂ + CO₂ \rightarrow CH₄ + 2 H₂O C₅H₁₂O₆ + 6 O₂ \rightarrow 6 CO₂ + 6 H₂O glucose C₅H₁₂O₆ \rightarrow 2 CH₃CHOHCOOH

 $\begin{array}{c} \textit{lactic acid} \\ \text{CH}_3\text{COOH} + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{CH}_3\text{CH}_2\text{COOH} + \text{H}_2\text{O} \\ \textit{acetic acid} & \textit{ethanol} & \textit{butyric acid} \end{array}$

cetic acia ethanol butyric acid $CH_2NH_2COOH + CH_3CHNH_2COOH + H_2O \rightarrow CH_3COOH + CH_3COCOOH + 2 NH_3 glycine alanine pyruvic acid$

substance other than oxygen serves as the hydrogen acceptor, the reaction is anaerobic. Anaerobic oxidation-reductions are often referred to as fermentations. Although the presence or absence of oxygen is of critical importance in the metabolism of many bacteria, the basic mechanisms of aerobic and anaerobic respiration are essentially the same.

Energy sources for bacteria. Bacteria make use of a far wider variety of energy sources than any other group of organisms. Photosynthetic bacteria utilize the energy of visible light, chemo-autotrophic organisms oxidize inorganic compounds as their sole source of energy, and heterotrophic bacteria oxidize almost every known type of organic compound. A few representative examples are listed in 2. In each case, the indicated reaction furnishes all the energy needed for growth of that species. The carbohydrates are the most widely utilized energy sources for heterotrophs, but acids such as succinic and lactic acids, the fatty acids, and the monohydroxy alcohols are also oxidized by many different bacteria. Most bacteria can use several different compounds as the main source of energy for metabolism and growth, some as many as a hundred. A few, however, have specific energy requirements. Thus, Clostridium kluyveri grows only when it can oxidize ethanol, while Nitrosomonas europaea must oxidize ammonia.

Mechanism of biological oxidation. Bacteria actually do not obtain energy from the substance oxidized in a single step, as suggested by the equations of table 2. These equations merely represent the overall process of biological oxidation and are the summation of many separate, consecutive oxidation-reductions, each catalyzed by specific respiratory enzymes. Consider the general equation for aerobic oxidation just written. In most bacteria, such a reaction involves at least the steps shown in 3 below.

Such a stepwise oxidation allows the cell to utilize the energy released in the oxidation in the most efficient manner possible and is characteristic of the reactions of intermediary metabolism in general. The chemical nature and detailed function of the respiratory enzymes involved will be considered later, but it should be noted here

- (1) Each respiratory enzyme exists in both an oxidized and a reduced state.
- (2) The respiratory enzymes enter into reversible oxidation-reduction reactions but are not used up in the overall process. They function catalytically as intermediate hydrogen carriers.
- (3) The enzyme that oxidizes the energy source (the *substrate*) cannot directly transfer the hydrogen and electrons of the substrate to oxygen, while the enzyme that finally does react with oxygen cannot oxidize the substrate. Therefore, the functioning of the complete chain of respiratory enzymes is essential for aerobic oxidation.

In most aerobic organisms this system is complete and functional, but in facultative and obligate anaerobes, portions of the hydrogen transport chain may be absent or nonfunctional (see below). In anaerobic respiration, the hydrogen and electrons of substrates are not transferred to molecular oxygen but to other substrates instead.

RESPIRATORY ENZYMES IN BACTERIA10

Dehydrogenases. 3 Dehydrogenases consist of a specific protein, or apoenzyme, combined with a reversibly oxidized and reduced coenzyme. They oxidize metabolites and are in turn reoxidized by other enzymes, not by molecular oxygen. Dehydrogenases possess a high degree of specificity for the substrate they oxidize

(1) A-H₂ + pyridinoprotein \rightleftharpoons A + pyridinoprotein-H₂ (2) pyridinoprotein-H₂ + flavoprotein \rightleftharpoons pyridinoprotein + flavoprotein-H₂ (3) flavoprotein-H₂ + 2 cytochrome Fe⁺⁺⁺ \rightleftharpoons flavoprotein + 2 cytochrome Fe⁺⁺⁺ + 2 H⁺ (4) 2 cytochrome Fe⁺⁺ + 2 cytochrome oxidase Fe⁺⁺⁺ \rightleftharpoons 2 cytochrome Fe⁺⁺⁺ + 2 cytochrome oxidase Fe⁺⁺ (5) 2 cytochrome oxidase Fe⁺⁺ + $\frac{1}{12}$ O₂ + 2 H⁺ \rightleftharpoons 2 cytochrome oxidase Fe⁺⁺⁺ + H₂O

^{3.}

and are usually named on the basis of their substrate specificity, *i.e.* lactic dehydrogenase, succinic dehydrogenase, etc. This substrate specificity is a function of the protein component, since a single coenzyme may be a part of several different dehydrogenases. The specific protein combines reversibly with both coenzyme and substrate, and the actual oxidation-reduction probably occurs while all three are in physical combination.

Two general types of dehydrogenases are found in bacteria and in other organisms. The *pyridinoproteins* are dehydrogenases containing either nicotinamide-adenine dinucleotide (NAD) or nicotinamide-adenine dinucleotide phosphate (NADP) as coenzymes. ¹⁶ The provisional structural formula of NAD is shown in 4. The structure of NADP is identical with that of NAD except for the presence of an additional molecule of phosphoric acid as shown in 4. Only the pyridine ring of both NAD and NADP undergoes reversible oxidation-reduction as indicated in the accompanying equation (5).

The pyridinoproteins are soluble, extracted from cells with relative ease, and their reduced forms are reoxidized by flavoproteins. In bacteria, the malic, lactic, and 3-phosphoglyceraldehyde dehydrogenases are NAD-proteins, while glutamic acid dehydrogenase is an NADP-protein. Many other bacterial dehydrogenases are also pyridinoproteins.

The cytochrome-linked dehydrogenases are associated with the insoluble particles of the cell. They were once thought to transfer the hydrogen of their substrates directly to the cytochrome system, hence their name. However, these enzymes are not single enzymes, but a complex of them. For example, succinic dehydrogenase, the most completely characterized complex of this nature, consists of at least the following components: (1) an iron-flavoprotein which oxidizes succinic acid to fumaric acid, (2) Slater's factor, (3) cytochrome c-linking factor, and (4) cytochrome b. These components react in a sequence, as yet not completely established, to transfer electrons from succinic acid to cytochrome c with the net result that succinic acid is oxidized and cytochrome c is reduced. Other microbial enzymes of a similar nature are the lactic dehydrogenase of yeast and the formic dehydrogenase of Escherichia coli.

Ferredoxin.³⁸ This is an iron-containing protein that functions as an electron carrier in anaerobic and photosynthetic bacteria. Depending on the species of origin, it contains four to seven molecules of nonheme iron per molecule of about 6000 molecular weight. Although it is a protein, it functions as a coenzyme of electron transport in a manner comparable to that of the pyridine nucleotides. The dark brown oxidized form of ferredoxin is oxidized to the reduced colorless state by appropriate enzyme systems such as the clostridial pyruvate dehydrogenase. It is reoxidized by other enzymes such as pyridine nucleotide reductase. Electron transport by ferredoxin plays a

nicotinamide-adenine dinucleotide (NAD)

* Position of third NADP phosphate group.

5.

O

C-NH₂

$$+2H^{+}+2e \rightleftharpoons N + H^{+}$$

ribose-R

oxidized form

 $+2H^{+}+2e \rightleftharpoons N + H^{+}$

ribose-R

role in biological nitrogen fixation, bacterial photosynthesis, and numerous substrate oxidations by anaerobes.

Flavoproteins. 11 The flavoproteins are respiratory enzymes containing either riboflavin phosphate or flavin adenine dinucleotide as coenzymes (see 6).

In both riboflavin coenzymes, oxidationreduction is confined to the flavin portion of the molecule (see 7). Many flavoproteins contain one or more atoms of iron, molybdenum, or copper. The metals apparently facilitate the transfer of electrons from the reduced flavin coenzyme to the cytochrome system. Derivatives of riboflavin are present in all bacteria and are found in especially large amounts in some anaerobes. Some flavoproteins oxidize substrates with the formation of hydrogen peroxide (8). It is generally believed that such flavoprotein oxidases are usually responsible for the formation of hydrogen peroxide in bacteria. Other flavoproteins also oxidize substrates but transfer electrons to oxygen by way of the cytochrome system. Still others function in the chain of electron transport by oxidizing reduced pyridine nucleotides and then reducing cytochrome. These enzymes are called cytochrome reductases or dia-

flavin adenine dinucleotide

phorases. Flavoproteins of all three types are of apparently general occurrence in bacteria.

Oxygenases.¹³ Like dehydrogenases, oxygenases are oxidative enzymes that react directly with substrates. Unlike dehydrogenases, which catalyze the transfer of electrons and hydrogen from substrates to suitable acceptors, oxygenases catalyze the incorporation of molecular oxygen into substrates. These enzymes play an important role in the synthesis and degradation of amino acids, sugars, fats, B vitamins, and other essential metabolites.

Dioxygenases catalyze the incorporation of two atoms of oxygen into one molecule of substrate as in 9a. The enzyme that catalyzes this reaction, metapyrocatechase, has been crystallized from a member of the genus Pseudomonas. It contains one atom of Fe⁺⁺ per molecule. Pyrocatechase is also a dioxygenase from a pseudomonad, and it catalyzes a similar reaction (9b). However, it contains one molecule of Fe⁺⁺⁺ per molecule.

Monoxygenases transfer only one atom of oxygen to each substrate molecule, as in the oxidation of lysine by lysine oxygenase (10). This enzyme contains two moles of flavin adenine dinucleotide per molecule and no detectable heavy metals.

Iron-porphyrin protein enzymes.³⁶ Several important respiratory enzymes have ironporphyrin compounds as coenzymes. These iron-porphyrins are closely related to, but not identical with, the heme of hemoglobin. Only the iron atom of the iron-porphyrin undergoes reversible oxidation-reduction. Cells of higher organisms contain at least three iron-porphyrin protein pigments, the cytochromes a, b, and c, and other similar proteins. The cytochromes are usually, but not always, present in aerobic bacteria and absent in obligate anaerobes. Bacterial cytochromes differ from those of plant and animal tissues in absorption spectra, specificity, and other physical and chemical characteristics. Bacterial cytochromes are remarkable for their variety, both within a single species and among different species. The cytochromes take part in electron transport by accepting electrons from cytochrome reductase and passing them on to cytochrome oxidase.

Cytochrome oxidase, Warburg's respiratory enzyme, is a portion of the sub-

10.

$$\begin{array}{cccc} CH_2NH_2 & CHNH_2 \\ CH_2 & CH_2 \\ CH_2 & CH_2 \\ CH_2 & CH_2 \\ CHNH_2 & CH_2 \\ CHNH_2$$

microscopic particulate structure of the cell, but it may be brought into solution or stable suspension by treatment with ultrasonic vibrations. It is also an iron-porphyrin protein. Cytochrome oxidase is the enzyme which reduces molecular oxygen to H₂O to complete the last link in the chain of aerobic respiration. The identity and number of cytochromes functioning between flavoprotein and cytochrome oxidase is not known for most bacteria. More than one oxidase may be present in a single cell.

A few bacteria, such as *Streptococcus* fecalis and *Lactobacillus* delbrueckii, carry out aerobic oxidations in the complete absence of cytochromes or cytochrome oxidase. In these organisms, the respiratory enzyme that transfers electrons to molecular oxygen is a flavoprotein.

Most organisms contain an enzyme called *catalase* which decomposes hydrogen peroxide.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The catalase crystallized from beef liver is an iron-porphyrin protein, and it has been generally accepted that catalase from other sources has a similar chemical nature, but recent evidence suggests that catalases other than iron-porphyrins may be present in some bacteria. Catalase is present in almost all aerobic bacteria and some facultative anaerobes, but under usual conditions of culture it cannot be detected in clostridia and some of the streptococci, lactic acid bacteria, and dysentery bacteria.

The *peroxidases* are enzymes, usually iron-porphyrin proteins but sometimes flavoproteins, which catalyze the oxidation of substrates by hydrogen peroxide. Their distribution in bacteria parallels that of the cytochromes and catalase, and their function in bacterial metabolism is uncertain.

THE RELATION OF BACTERIA TO MOLECULAR OXYGEN

The bacteria differ from one another in their relationship to molecular oxygen. Certain bacteria, the *obligate aerobes*, require ready access to air, growing feebly or not at all in its absence. Such organisms may lack certain respiratory enzymes necessary for anaerobic respiration, or the end products of their anaerobic metabolism may be toxic to them. The exact cause of their obligate oxygen requirement remains unknown. This group includes such well-known bacteria as the nitrifying bacteria, some of the sulfur bacteria, *Bacillus subtilis* and related forms, Azotobacter, the diphtheria bacillus, the cholera vibrio, and others.

However, most bacteria can grow in the complete or virtual absence of molecular oxygen. Two types of anaerobic bacteria may be distinguished. The most numerous are the facultative anaerobes, which respire with equal facility in the presence or absence of molecular oxygen. In broth cultures the dissolved oxygen is soon exhausted, and, after a preliminary period of aerobic growth, the culture continues to develop under essentially anaerobic conditions. The obligate anaerobes are unable to grow in the presence of molecular oxygen, and oxygen is actively toxic to them; vegetative cells die very quickly upon exposure to air, although spores are highly resistant. Members of the genus Clostridium, together with certain micro-aerophilic forms which tolerate very low concentrations of oxygen, are usually included in this group.

Bacteria are unexcelled in the possession of powerful and varied means for obtaining energy from nutrients in the absence of oxygen. The energy obtained from a given quantity of nutrients is usually much less under anaerobic conditions than in the presence of oxygen, because oxidation of substrates is less complete, and anaerobic metabolism is characterized by a rapid utilization of oxidizable substrates and a great accumulation of partially oxidized end products.

Bacteria perform energy-yielding oxidations anaerobically by using both inorganic and organic compounds as hydrogen acceptors instead of molecular oxygen. Thus, *E. coli* contains respiratory enzymes which couple the oxidation of lactic acid with the reduction of nitrate (see 11).

Bacteria also frequently couple oxidation of one organic compound with the reduction of a second. In this manner, glyceraldehyde may be oxidized by the enzymatic transfer of hydrogen to pyruvic acid (see 12).

This type of reaction is the chief mode of oxidation-reduction in the fermentation of carbohydrates, the chief anaerobic energy source for the great majority of bacteria. However, certain of the obligate anaerobes possess only a very limited ability to metabolize carbohydrate, and an important source of energy for these bacteria appears to be the coupled oxidation-reduction of pairs of amino acids.

CONSERVATION AND TRANSFER OF THE ENERGY RELEASED IN BIOLOGICAL OXIDATIONS^{17, 31}

So far nothing has been said of how energy released in respiration is conserved and made available for synthetic purposes. Yet these processes are as important as the energy-yielding reactions themselves, since energy simply liberated as heat (as in oxidation carried out in the test tube by the organic chemist) is not available for synthesis of new cell constituents.

The general principles involved are well illustrated by a reaction already discussed, the coupled oxidation-reduction of glyceraldehyde and pyruvic acid. Our present concepts of energy transfer in biological systems arose from detailed studies of this key reaction in carbohydrate fermentation (see below). Actually several different enzymes and coenzymes participate in this complicated reaction, but for the purposes at hand, it may be represented as shown in 13. Note that both inorganic and organic forms of orthophosphoric acid (H₃PO₄)

participate in the reaction. This is because the inorganic and organic phosphate compounds form an integral part of the energyconserving and transferring mechanism.

The key to the role of phosphate in energy metabolism lies in the difference between the two kinds of phosphate groups present in 1, 3-diphosphoglyceric acid. Let us see what this difference is and wherein lies its significance for biological oxidation.

1,3-diphosphoglyceric acid

Phosphate group A

 The phosphate group is united with the rest of the molecule through an ester linkage with a primary alcohol group.

(2) By analogy with simple and familiar organic compounds, this linkage may be compared with the ester bond joining ethanol and acetic acid in ethyl acetate.

(3) Like ethyl acetate, this *phosphate ester* is not easily hydrolyzed and liberates but little heat (energy) on hydrolysis.

(4) For these reasons, organic phosphate esters, as well as inorganic phosphate salts, are said to

12.

$$\begin{array}{ll} \text{CH}_2\text{OH-CHOH-CHO} + \text{CH}_3\text{-CO-COOH} + \text{H}_2\text{O} \rightleftharpoons \text{CH}_2\text{OH-CHOH-COOH} + \text{CH}_3\text{-CHOH-COOH} \\ \textit{glyceraldehyde} & \textit{pyruvic acid} & \textit{glyceric acid} & \textit{lactic acid} \end{array}$$

contain low-energy phosphate bonds, which are often designated by the shorthand notation, R-P.

Phosphate group B

(1) The phosphate group is united with the rest of the molecule through an anhydride linkage to a carboxyl group.

(2) By analogy, this bond may be compared to the anhydride bond between two molecules of acetic acid in acetic anhydride.

(3) Like acetic anhydride, this *phosphate anhydride* is readily hydrolyzed and liberates large amounts of heat (energy) on hydrolysis.

(4) Organic phosphate anhydrides are said to contain high-energy phosphate bonds, which are represented as R~P.

When the oxidation of 3-phosphoglyceraldehyde is coupled with the uptake of inorganic phosphate to form 1, 3-diphosphoglyceric acid, the energy liberated as heat is very much less than the heat released in the simple oxidation of glyceraldehyde to glyceric acid. The reason for this difference should now be apparent: the missing energy has been trapped and conserved in the form of the high-energy phosphate bond of 1, 3-diphosphoglyceric acid. The detailed reaction mechanism is shown in 14. All three steps are catalyzed by 3-phosphoglyceralde-

hyde dehydrogenase. 3-phosphoglyceraldehyde first combines with the thiol (SH) group of the enzyme to form a thiohemiacetal which is oxidized to a thioacyl group with NAD as the hydrogen acceptor. Finally in step (c), the thioacyl group is cleaved by the addition of inorganic phosphate to yield 1, 3-diphosphoglyceric acid and to regenerate the dehydrogenase in its SH form. Such a coupling of oxidation with the uptake of low-energy inorganic phosphate into highenergy organic phosphate compounds appears to be the chief method of energy conservation in all living cells. Therefore, representation of the general reaction of biological oxidation may be broadened to include the energy-conserving mechanism:

$$P + A - H_2 + B \rightleftharpoons A \sim P + B - H_2$$

(inorganic) (high energy)

There remains another problem—how the energy conserved in high-energy phosphate bonds is transferred to the appropriate place in the metabolic machinery where it may be used up in energy-requiring syntheses. Using the same example as before, this is what occurs:

1,3 diphosphoglyceric acid + adenosine diphosphate
 ⇒
 3-phosphoglyceric acid + adenosine triphosphate

Adenosine diphosphate (ADP) and adeno-

jalite Sottatian kaj etchologi Sama kaloniski kalonio Amelono 114

NH2

O O O

CH2-O-P-O
$$\sim$$
P-OH

OH OH OH

 $Adenosine\ triphosphate\ (ATP)$
 $+$ P $\uparrow \mid -$ P

NH2

C N

CH2-O-P-O \sim P-OH

OH OH

 $Adenosine\ diphosphate\ (ADP)$

sine triphosphate (ATP) have the structure

shown in 15.

Note that ATP has two energy-rich bonds while ADP has only one, which is not usually active in energy transfer. This is the only difference between the two substances. All cells appear to have enzymes which reversibly convert one to the other by the addition or removal of an energy-rich bond. The resemblance of this reversible system to the reversibly oxidized and reduced hydrogen carriers is very striking. By analogy they may be called intermediate energy carriers or energy donors and energy acceptors, and this is exactly how they function in metabolism. ATP and ADP form the connecting

link between the energy-yielding reactions of respiration and the energy-demanding reactions of synthesis (see 16).

In subsequent sections, it will be seen that many types of oxidations, aerobic and anaerobic, inorganic and organic, generate ATP and that the energy stored in ATP is used in almost every type of biological synthesis. High-energy phosphate bonds are generated, not only during oxidations at the substrate level, but also in the oxidations accompanying the further steps in aerobic respiration. The steps in electron transfer implicated in oxidative phosphorylation are the reoxidation of NADH and reduced cytochrome.

Carbohydrate Metabolism

Carbohydrates serve a dual function in the metabolism of heterotrophic bacteria: (1) they are oxidized as the chief source of energy (ATP and reduced pyridine nucleotide) in most species and (2) they are the principal, and often the only, source of carbon for the synthesis of bacterial carbohydrates, lipids, amino acids, purine and pyrimidine bases, and all other organic cell constituents. In practice, these functions are inseparable. The metabolic transformations necessary for the assimilation of carbohydrates into cell material are often

also energy-yielding breakdowns. In addition, almost all the reactions involved in the degradation of carbohydrates are metabolically reversible and thus are also used for synthesis of new carbohydrates from small molecules such as CO₂, acetic acid, and ethanol.

Bacteria form a bewildering variety of products from carbohydrates, but their metabolism proceeds along a relatively few well-defined pathways of wide distribution in bacteria and in all other living things as well. Therefore variation in me-

tabolism among different species is not so much the result of fundamental differences in mode of metabolism as of differences in the relative importance of the several possible common pathways.

METABOLISM OF COMPLEX CARBOHYDRATES

Carbohydrates composed of two or more monosaccharide units linked together by glycosidic bonds are called complex sugars. Those with a definite small number of monosaccharide units are called oligosaccharides; most of them are disaccharides, such as maltose which is composed of two molecules of glucose. Polysaccharides contain a large and indefinite number of monosaccharide units. For example, the amylose fraction of starch contains molecules made up of straight chains of 300 to 1000 glucose units.

Hydrolysis of complex sugars.³⁵ Although free monosaccharides are widely distributed in nature, by far the greater bulk of naturally occurring carbohydrates are oligosaccharides and polysaccharides. With few exceptions bacteria must first break down the complex sugars into their constituent monosaccharides before they can be used as energy sources or as raw material for the synthesis of new protoplasm. This is done by cleaving the glycosidic bond uniting two monosaccharide units.

Bacteria break down complex sugars by three principal mechanisms, all of which may be illustrated with one sugar, sucrose. The examples in 17 give both the reaction and a typical enzyme which catalyzes it.

As will be seen in the section immediately following, the last two kinds of enzymes may also synthesize oligosaccharides and polysaccharides. However, hydrolysis of compound sugars is a virtually irreversible process and plays no important part in their synthesis. Nevertheless, bacteria possess a formidable battery of hydrolytic enzymes which are of great importance to the bacterium in utilizing the carbohydrate foodstuffs of its environment. They are of equal importance to the bacteriologist in classifying, identifying, and differentiating bacteria, because the presence or absence of enzymes hydrolyzing complex sugars largely determines the fermentation reactions of different species. Some of these enzymes are synthesized by the bacterial cell whether their substrate is present or not (constitutive enzymes), while others are produced only in the presence of the appropriate substrate (induced enzymes).

Relatively few species of bacteria are able to decompose *cellulose*, but those that do are widely distributed in the soil and the mud of sea bottoms as well as in the intestines of herbivorous animals. Cellulose is hydrolyzed to glucose, sometimes directly, and sometimes with the intermediate formation of the disaccharide cellobiose.

Unlike cellulose, *starch* may be hydrolyzed by a wide variety of living organisms, and the extracellular enzyme diastase, or amylase, is possessed by many bacteria.

16.

$$\left. \begin{array}{l} \text{energy-yielding oxidations} \\ \text{supply} \sim P \end{array} \right\} \\ ADP \frac{+ \sim P}{- \sim P} \\ ATP \\ \begin{array}{l} \text{energy-requiring syntheses} \\ \text{consume} \sim P \end{array}$$

17.

Breakdown of Complex Sugars

(1) Hydrolysis

sucrose + H₂O
$$\xrightarrow{\text{invertase}}$$
 glucose + fructose

(2) Phosphorolysis

sucrose + H₂PO₄ $\xrightarrow{\text{sucrose}}$ glucose-1-P + fructose

(3) Transglycosidation

dextran

n sucrose $\xrightarrow{\text{dextran}}$ (anhydroglucose)_n + n fructose

sucrase $\xrightarrow{\text{dextran}}$

Like cellulose, however, starch is hydrolyzed in at least two stages, first to the disaccharide maltose, and second by a further hydrolysis to glucose.

Many disaccharides are also hydrolyzed by bacterial enzymes; sucrose by invertase, maltose by maltase, cellobiose by cellobiase,

lactose by β -galactosidase, etc.

Synthesis of complex sugars.¹⁵ The essential reaction in the synthesis of oligosaccharides and polysaccharides is the formation of the glycosidic bond. This reaction may be studied in its simplest form in the synthesis of disaccharides. Sucrose is formed from glucose-1-phosphate and fructose by the action of the enzyme sucrose phosphorylase found in *Pseudomonas saccharophila*, *Leuconostoc mesenteroides*, and other bacteria (see 17 [2]).

In contrast to the *hydrolysis* of sucrose by invertase, the *phosphorolysis* of sucrose by sucrose phosphorylase is reversible, and this enzyme readily synthesizes the disaccharide. This is because it is very hard to eliminate a molecule of water from the two monosaccharides in aqueous solution, while it is relatively easy to split out a molecule of phosphoric acid.

In syntheses catalyzed by enzymes of the

sucrose phosphorylase type, the problem of glycosidic bond synthesis becomes one of glucose-1-phosphate production. The formation of glucose-1-phosphate and its utilization for disaccharide synthesis is a good example of how the energy stored in ATP by oxidative reactions is used for synthetic purposes (18). Once glycosidic linkages are formed, other complex sugars may be formed by the transfer of monosaccharide units, or transglycosidation. Again, this mechanism may be illustrated with sucrose:

glucosido-fructoside + sorbose ⇒ (sucrose)

glucosido-sorboside + fructose

Note that the *number* of glycoside linkages remains the same. The glucose unit has merely been transferred from glycosidic linkage with fructose to glycosidic linkage with sorbose.

Other transglycosidases catalyze the formation of polysaccharides. Thus, the dextran sucrase of *Leuc. mesenteroides* forms from sucrose the polysaccharide dextran, which is composed of many glucose units (see 17 [3]).

Another type of transglycosidation in-

18.

glucose + ATP \rightarrow glucose-6-P + ADP glucose-6-P \rightleftharpoons glucose-1-P glucose-1-P + fructose \rightleftharpoons sucrose + H₃PO₄

Net reaction: glucose + fructose + ATP → sucrose + ADP + H₃PO₄

19.

uridine diphosphate glucose

volves the participation of a derivative of the pyrimidine uracil, uridine diphosphate glucose (19), as the monosaccharide unit donor. Yeast contains an enzyme which synthesizes sucrose according to the equation:

uridine diphosphate glucose + fructose ⇒ uridine diphosphate + glucosido-fructoside (sucrose)

Uridine diphosphate glucose is regenerated as follows:

uridine diphosphate + glucose-1-P→ uridine diphosphate glucose + P

If the examples discussed here are representative of the mechanisms responsible for complex sugar synthesis in all bacteria, it may be concluded that bacteria do not form oligosaccharides and polysaccharides by directly combining simple monosaccharides. Instead, they (1) from glucose-1phosphate or analogous compounds with the energy of ATP, (2) form initial glucosidic bonds by splitting out H₃PO₄ between glucose-1-phosphate and another monosaccharide or uridine diphosphate, and (3) form other disaccharides and polysaccharides by the transglycosidation reaction.

Great progress has been made in recent years in understanding how polysaccharides with more than one kind of monosaccharide unit are synthesized. For example let us consider the biosynthesis of hyaluronic acid, a polysaccharide occurring both in bacteria and in higher animals and of probable importance in certain bacterial infections. Hyaluronic acid is composed of a large number of basic units consisting of a molecule of glucuronic acid and one of Nacetylglucosamine (20). It is synthesized as shown in 21.

A number of important bacterial polysaccharides are synthesized by analogous mechanisms. 15 Among them are the polysaccharide moiety of murein, the structural polymer of the bacterial cell wall,27 the lipopolysaccharides of gram-negative bacteria, the teichoic acids of gram-positive bacteria, and the type-specific capsular polysaccharides of pneumococci.

BREAKDOWN OF HEXOSES AND PENTOSES

This topic is most simply presented by considering first the degradation of glucose. Since pentoses are frequent early products of glucose catabolism, the breakdown of pentoses is also discussed here. Finally, the way in which other common hexoses and hexose derivatives are channeled into the pathways of glucose metabolism will be shown.

Basic unit of hyaluronic acid

21.

(a) uridine diphosphate glucose + 2 NAD → uridine diphosphate glucuronic acid + 2 NADH

glucose-6-P → fructose-6-P fructose-6-P fructose-6-P + glutamine → glucosamine-6-P + glutamate glucosamine-6-P + acetyl-coenzyme A → N-acetyl-glucosamine-6-P + coenzyme A N-acetyl-glucosamine-6-P → N-acetyl-glucosamine-1-P

N-acetyl-glucosamine-1-P + uridine triphosphate -> uridine diphosphate-N-acetyl-glucosamine-1-P + P-P

(c) uridine diphosphate-N-acetyl-glucosamine-1-P + uridine diphosphate glucuronic acid → hyaluronic acid + 2 uridine diphosphate + P Diagram 22 outlines the four principal known pathways of glucose breakdown in bacteria. It should be used only as a guide to the detailed discussion of these pathways, for in order to show all possible modes of glucose degradation in a single diagram, certain simplifications have been made and alternate variations within a single pathway have been omitted.

All the mechanisms for breakdown of glucose may be roughly divided into three stages:

- (1) Conversion of glucose, by phosphorylation, oxidation, or by both processes, to a 6-carbon sugar which is the substrate for stage 2.
- (2) Cleavage of the carbon skeleton of the 6-carbon intermediate formed in stage 1 to two 3-carbon fragments or to a 5-carbon sugar (pentose) and CO₂.
- (3) Further metabolism of the reaction products of stage 2 to pyruvic acid (note exception in pathway 4).

The fate of pyruvic acid, the common product of all four pathways, will be the subject of the section immediately following.

Pathway 1: Nonphosphorylating oxidation of glucose. 14 Originally described in pseudomonads and acetic acid bacteria, these reactions have now been found in many other gram-negative and gram-positive bacteria.

The unique step in this mechanism is the direct oxidation, without prior phosphorylation, of glucose to gluconic acid. Further steps vary in different bacteria. An example of the pathway from glucose to pyruvic acid in *Ps. fluorescens* is shown in 23.

It is believed that considerable useful energy may be generated by means of phosphorylations coupled with the oxidation of glucose to 2-ketogluconate.

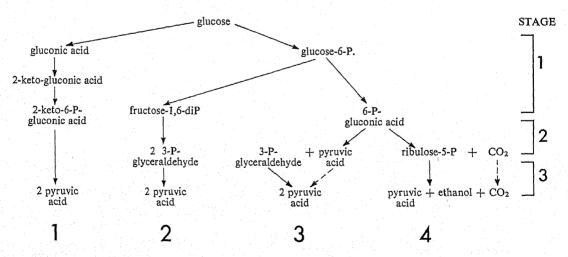
Pathways 2, 3, and 4: Formation of glucose-6-phosphate as a common initial step. Glucose is metabolized by way of the three remaining pathways by being first phosphorylated to glucose-6-phosphate by ATP and the enzyme hexokinase.

Pathway 2: Phosphorylating glycolysis.² Breakdown of glucose by phosphorylating glycolysis, the Embden-Meyerhof scheme, occurs in many bacteria, sometimes as the sole means of glucose breakdown, as in the homofermentative lactic acid bacteria, but more frequently in combination with other pathways.

The transformation of glucose to pyruvic acid by way of the Embden-Meyerhof scheme may be considered to proceed in four stages (summarized in 24).

22.

Breakdown of Glucose



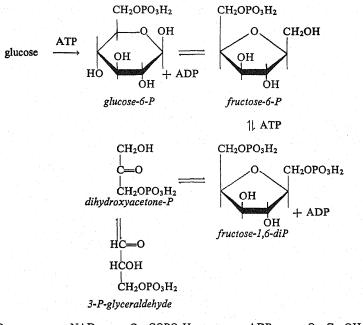
PATHWAYS

(numbered for convenience of discussion)

24.

2

Phosphorylating Glycolysis of Glucose to Pyruvic Acid



HC=0NADO=COPO3II2ADPO=C—OHHCOH
$$H_3PO_4$$
HCOHHCOH+ ATP $CH_2OPO_3H_2$ $CH_2OPO_3H_2$ $CH_2OPO_3H_2$ 3-P-glyceraldehyde $I,3$ -diP-glyceric acid3-P-glyceric acidO=C—OHO=C—OHO=C—OHO=C—OHHCOH $HCOPO_3H_2$ $COPO_3H_2$ $COPO_3H_2$ $CH_2OPO_3H_2$ CH_2OH CH_2OH CH_2OH 3-P-glyceric acid CH_2OH CH_2OH CH_2OH 3-P-glyceric acid CH_2OH CH_2OH CH_2OH

(1) Formation of fructose-1,6-diphosphate. The hexose actually split to triose is fructose-1,6-diphosphate, formed as follows:

$$\begin{array}{c} \text{isomerase A} \\ \text{glucose-6-P} & \longrightarrow \\ \text{fructose-6-P} \end{array}$$

(2) Cleavage of fructose-1,6-diphosphate to triose. Fructose-1,6-diphosphate is split into two trioses, which are enzymatically interconvertible.

The occurrence of this aldolase reaction may be considered the unique reaction of the Embden-Meyerhof pathway.

(3) Oxidation of 3-phosphoglyceraldehyde. Of the two trioses formed in stage 2, only 3-phosphoglyceraldehyde is oxidized under normal conditions. The reaction is catalyzed by 3-phosphoglyceraldehyde dehydrogenase which contains NAD as a coenzyme. The detailed mechanism of this reaction has already been described.

The high-energy phosphate group of 1,3-diphosphoglyceric acid is transferred to ADP under the influence of another enzyme:

Finally, the reduced NAD is reoxidized by an appropriate hydrogen acceptor and its dehydrogenase. In the lactic fermentation, the reaction is:

$$pyruvic \ acid + \ NADH + \ H^{+} \ \frac{lactic}{dehydrogenase}$$

lactic acid + NAD+

In alcoholic fermentation, acetaldehyde is reduced to ethanol (see section on bacterial fermentations).

(4) Formation of pyruvic acid. 3-phosphoglyceric acid is rearranged to 2-phosphoglyceric acid:

Then 2-phosphoglyceric acid undergoes an intermolecular oxidation-reduction to phosphoenolpyruvic acid:

$$CH_{2}OH - C - COOH \xrightarrow{enolase}$$

$$CH_{2}OH - C - COOH \xrightarrow{enolase}$$

$$O \sim PO_{3}H_{2}$$

$$CH_{2} - COOH + H_{2}O$$

The formation of phosphoenolpyruvic acid is another example of how energy released in oxidations is conserved in high-energy phosphate bonds. Here the low-energy phosphate group of 2-phosphoglyceric acid is converted into the high-energy group of phosphoenolpyruvic acid.

In the final pyruvate-forming reaction, the high-energy bond of phosphopyruvate is enzymatically transferred to ADP.

phosphoenolpyruvic acid + ADP ⇒ pyruvic acid + ATP

Since many bacteria such as the homofermentative lactobacilli gain their energy from anaerobic oxidation of glucose to lactate, there must be a net production of energy in the process. A balance sheet in loss and gain of ATP makes this clear.

ATP used up per mole of glucose

- 1 ATP used for formation of glucose-6-phosphate from glucose
- 1 ATP used for formation of fructose-1,6-diphosphate from fructose-6-phosphate

Total 2 ATP used up

ATP formed per mole of glucose

- 2 ATP formed from oxidation of 3-phosphoglyceraldehyde to 3-phosphoglyceric acid (1 glucose → 2 glyceraldehyde)
- 2 ATP formed from transformation of 2-phosphoglyceric acid to phosphoenolpyruvic acid.

Total 4 ATP formed

Therefore, oxidation of one molecule of glucose to lactic acid results in the net formation of two moles of ATP.

Pathways 3 and 4: Formation of 6-phosphogluconic acid as a common step. Glucose-6-phosphate is oxidized to 6-phosphogluconic acid by dehydrogenases which in

most organisms require NADP as coenzyme (see 25).

Pathway 3: The Entner-Doudoroff pathway. 14 In many gram-negative bacteria, 6-phosphogluconic acid is cleaved to two 3-carbon compounds through the intermediate formation of 2-keto-3-deoxy-6-phosphogluconic acid. Thus, splitting of this compound identifies the Entner-Doudoroff pathway in the same way that the splitting of fructose-1,6-diphosphate identifies the Embden-Meyerhof scheme (see 26). The 3-phosphoglyceraldehyde may

then be converted to pyruvic acid by way of the reactions of pathway 2. The energy yield is one mole of ATP for each mole of glucose utilized.

Pathway 4: The pentose phosphate pathway. This pathway differs from the other three in that the carbon skeleton of glucose is split at two different places in two separate reactions. First, 6-phosphogluconic acid is cleaved into a molecule of CO₂ and one of pentose by 6-phosphogluconic acid dehydrogenase (see 27).

The next step is the cleavage of the pen-

ribose-5-P

xvlulose-5-P

ribulose-5-P

tose molecule by the enzyme transketolase as shown in 28. Its coenzyme diphosphothiamin (DPT) is firmly bound to the enzyme protein. The structure of DPT is given in 29, which also shows the structure of dihydroxyethyl-DPT, the active 2-carbon intermediate formed by reaction between pentose and transketolase.²³ DPT forms analogous compounds in several other important reactions soon to be discussed.

At this point, the metabolism of pentose diverges in two directions, depending on the fate of the dihydroxyethyl-DPT. One pathway is the formation of lactate, acetate and ATP (30). Subtracting the mole of ATP used to form glucose-6-phosphate gives a net yield of two moles of ATP per mole of glucose, the same as in phosphorylating glycolysis. The formation of acetyl-coenzyme A and acetylphosphate will be discussed in detail in a later section.

The other, more complicated pathway involves the conversion of three molecules of

pentose to one molecule of 3-phosphoglyceraldehyde and two molecules of hexose. The first step is the oxidation of three hexose molecules (C_6) to pentose (C_5) as shown in 27.

Second, two pentoses are cleaved by transketolase to dihydroxyethyl-DPT (C_2 -DPT) and 3-phosphoglyceraldehyde (C_3) as described in 28.

Third, in a reaction also catalyzed by transketolase, a molecule of dihydroxyethyl-DPT is transferred to the remaining pentose to form the 7-carbon sugar, sedoheptulose as shown in 31.

Fourth, the enzyme transaldolase splits the sedoheptulose into the tetrose, erythrose-4-phosphate, and an active 3-carbon intermediate (see 31), which is immediately condensed with a molecule of 3-phosphoglyceraldehyde to form fructose-6-phosphate (see 32).

Finally, the erythrose-4-phosphate and the remaining molecule of dihydroxyethyl-DPT

28.

$$\begin{array}{c} \text{CH}_2\text{OH} \\ \text{C}=\text{O} \\ \text{HCOH} \\ \text{HOCH} \\ \text{CH}_2\text{OPO}_3\text{H}_2 \\ xy|u|ose-5-P \end{array} \qquad \begin{array}{c} \text{CH}_2\text{OH} \\ \text{HOC} - \text{DPT} \\ \text{HCOH} \\ \text{HOCH} \\ \text{CH}_2\text{OPO}_3\text{H}_2 \\ \end{array} \qquad \begin{array}{c} \text{CH}_2\text{OH} \\ \text{HOCH} \\ \text{CH}_2\text{OPO}_3\text{H}_2 \\ \end{array} \qquad \begin{array}{c} \text{CHOH} \\ \text{DPT} \\ \text{dihydroxyethyl-DPT} \\ \text{dihydroxyethyl-DPT} \\ \text{HC}=\text{O} \\ \text{HCOH} \\ \text{CH}_2\text{OPO}_3\text{H}_2 \\ \end{array}$$

29.

H

dihydroxyethyl-DPT

are condensed by transketolase to produce a second mole of fructose-6-phosphate (see 33).

The net reaction is:

3 glucose → 3 CO₂ + 3-P-glyceraldehyde +

2 fructose-6-P

The 3-phosphoglyceraldehyde may be me-

tabolized anaerobically according to the fermentation pattern of the bacterium involved or may be completely oxidized by way of pyruvic acid and the Krebs cycle (see below). The fructose-6-phosphate may be aerobically repassed through the pentose cycle or anaerobically degraded by any of the mechanisms already discussed. The energy yield will depend upon the precise

30.

- (a) xylulose-5-P + DPT \to dihydroxyethyl-DPT + 3-P-glyceraldehyde (b) dihydroxyethyl-DPT + coenzyme A \to acetyl-coenzyme A + DPT
- (acetyl-coenzyme A + H₃PO₄ → acetylphosphate + coenzyme A acetylphosphate + ADP → ATP + acetic acid
 (c) 3-P-glyceraldehyde + NAD+ + ADP + H₃PO₄ → 2-P-glyceric acid + ATP + NADH + H+ 2-P-glyceric acid + ADP → pyruvic acid + ATP pyruvic acid + NADH + H+ → lactic acid + NAD+

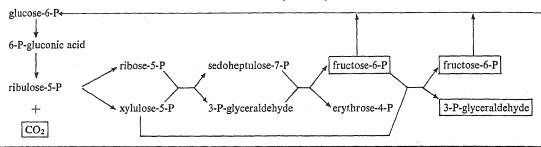
Net Reaction: xylulose-5-P + 2H₃PO₄ + 3ADP → acetic acid + lactic acid + 3ATP

31.

32.

34.

The Pentose Phosphate Cycle



fate of the hexose and triose produced by the pentose cycle. The pentose phosphate cycle is summarized in diagram 34.

Breakdown of other hexoses and pentoses. The many monosaccharides occurring in nature in either the free state or in oligosaccharides and polysaccharides are metabolized by conversion to intermediates in one or more of the pathways of glucose breakdown. Some typical conversions are:

fructose-6-P

fructose

ATP

fructose-1-P
$$\rightarrow$$

dihydroxyacetone-P + glyceraldehyde

mannose

ATP

mannose-6-P \rightarrow fructose-6-P

galactose

ATP

galactose-1-P \rightarrow

glucose-1-P \rightarrow glucose-6-P

Relative importance of different pathways of glucose metabolism.⁷ Considerable efforts have been made to determine the relative importance of the different pathways of glucose breakdown in specific organisms. Of

various methods used to estimate the amount of glucose broken down by each pathway. perhaps the most successful has been the use of glucose labeled with the radioactive isotope of carbon, C14. A simple example will illustrate the method. If glucose labeled in carbon 1 with C14 is degraded by pathways 2, 3, and 4, pyruvic acid may be a common product. However, the pyruvic acid formed by Embden-Meyerhof glycolysis contains the isotope in the methyl group of one of each two molecules formed. The pyruvic acid produced by the Entner-Doudoroff route contains C14 in the carboxyl group of one molecule, and the pyruvic acid formed by way of the pentose phosphate pathway contains none at all, because all the C14 was released as CO₂. Although the situation is by no means simple, quantitative studies of isotope distribution in the end products of glucose breakdown allow a reasonably reliable estimate of the relative importance of the various pathways. Some representative results are given in the accompanying table. In organisms exhibiting more than one mode of degradation (probably the major class), the exact relative proportions are dependent on many environmental conditions.

Pathways of Glucose Breakdown

ORGANISM	PER CENT GLUCOSE DEGRADED BY		
	CLYCOLYSIS	PENTOSE PHOSPHATE PATHWAY	ENTNER-DOUDOROFF PATHWAY
Lactobacillus casei Saccharomyces cerevisiae Escherichia coli Bacillus subtilis Leuconostoc mesenteroides Pseudomonas aeruginosa Pseudomonas saccharophila	100 88 72 59	12 28 41 100 29	71 100

BREAKDOWN OF PYRUVIC ACID

Bacteria dissimilate pyruvic acid by what appear to be many different mechanisms into the variety of end products which are characteristic of bacterial fermentations. There are common patterns in many apparently different modes of pyruvate breakdown which are probably of as regular and widespread occurrence as the Embden-Meyerhof glycolvsis.

Coenzymes of pyruvic acid metabolism. Three coenzymes are especially concerned in the metabolism of pyruvic acid: DPT, α -lipoic acid, and coenzyme A (CoA). Their constitution is indicated in the structural formulas in 29 and 35.

Diphosphothiamin¹⁶ is the coenzyme of the enzyme which catalyzes the decarboxylation of pyruvic acid to acetaldehyde. α-Hydroxyethyl-DPT is the primary product of pyruvate metabolism (see 36). It may be decomposed to yield free acetaldehyde or it may be further metabolized. Other α -keto acids, such as α -ketoglutaric

35.

α-Lipoic acid

pantothenic acid

Coenzyme A (CoA)

36.

acid, are usually decarboxylated by similar DPT-enzymes.

 α -Lipoic acid³² is a reversibly oxidized and reduced coenzyme which is the hydrogen acceptor in the oxidation of the acetaldehyde produced by decarboxylation of pyruvic acid (see 37). α -Lipoic acid is also a growth factor for both bacteria and protozoa.

Coenzyme A, ¹⁶ the acetylation coenzyme, is a complex structure composed of one molecule each of the growth factor pantothenic acid, adenylic acid, β -mercaptoethanolamine, and two molecules of phosphoric acid. It reacts with acetyl- α -lipoic acid to form acetyl-CoA (see 38). The reduced form of α -lipoic acid released in the reaction is reoxidized by an NAD-enzyme.

Reactions of pyruvic acid. (1) Reduction. In Embden-Meyerhof glycolysis, L(+)-lactic acid is formed from pyruvic acid by an NAD-specific lactic dehydrogenase. Other lactic dehydrogenases are found in organisms not containing the Embden-Meyerhof system. Leuc. mesenteroides has an NAD-dehydrogenase which forms the D(-) isomer of lactic acid from pyruvic acid. Some lactic dehydrogenases, such as that of L. delbrueckii, contain flavin nucleotides as coenzymes.

Pyruvic acid is also reduced to the amino

acid alanine in reactions of transamination (see below).

- (2) Nonoxidative decarboxylation and aldehyde transfer. These reactions have the same general mechanism and are catalyzed by DPT-containing enzymes. The first common step, the formation of α -hydroxyethyl-DPT, has already been described.
- (A) DIRECT DECARBOXYLATION. Yeast and many bacteria contain an enzyme, the classic pyruvic carboxylase of Neuberg, which directly decarboxylates pyruvic acid to free acetaldehyde and CO₂.

pyruvic acid + DPT-enzyme→
α-hydroxyethyl-DPT-enzyme + Co₂
α-hydroxyethyl-DPT-enzyme
acetaldehyde + DPT-enzyme→

The acetaldehyde may be reduced to ethanol, oxidized to acetic acid, or condensed with α -hydroxyethyl-DPT to form acetoin (see below).

(B) Transfer to acetalDeHyDE. Yeast, $E.\ coli$, and other organisms may transfer the α -hydroxyethyl group formed from pyruvic acid decarboxylation to a molecule of free acetaldehyde to form acetoin (see 39). Acetoin may be reduced to

37.

38.

CH₃—C—S O S
$$\alpha$$
-lipoic acid + CoA \rightleftharpoons CH₃—C—S—CoA + α -lipoic acid HS α -cetyl-CoA

39.

OH O O OH
$$CH_3-C-DPT + CH_3-C-H \rightarrow CH_3-C-C+CH_3 + DPT$$

$$H \qquad H$$

$$\alpha\text{-hydroxyethyl-DPT} \quad acetaldehyde \qquad acetoin$$

2,3-butanediol in some bacterial fermenta-

- (C) Transfer to Pyruvic acid. Another molecule of pyruvic acid may also accept the α -hydroxyethyl group to form acetoin by way of α -acetolactic acid (40).
- (3) Oxidative decarboxylation. The first step in both oxidative and nonoxidative decarboxylation of pyruvic acid is the formation of an acetaldehyde-DPT-enzyme complex. In the oxidative process, this intermediate is then transformed to a highly reactive form of acetic acid, acetyl-coenzyme A.

acetaldehyde-DPT +
$$\alpha$$
-lipoic acid \rightarrow acetyl- α -lipoic acid- H_2 + DPT

transacetylase acetyl-
$$\alpha$$
-lipoic acid- H_2 + CoA \Longrightarrow acetyl-CoA + α -lipoic acid- H_2

The energy made available by the oxidation of pyruvic acid to acetic acid is conserved in the form of acetyl- α -lipoic acid which, like acetyl-CoA, is a high-energy form of acetate.

The reduced lipoic acid is reoxidized by NAD (as already discussed). The NAD may then in turn be oxidized either aerobically or anaerobically.

Acetyl-CoA may also be formed from acetic acid. Two different modes of acetate activation are operative in bacteria.

acetyl-phosphate + ADP

$$\begin{array}{c} \text{acetyl-phosphate} + \text{CoA} & \xrightarrow{\text{phosphotransacetylase}} \\ & \text{acetyl-CoA} + \text{inorganic orthophosphate} \end{array}$$

In these reactions the energy of ATP is used to form an intermediate high-energy form of acetate which then reacts with CoA to form acetyl-CoA.

- (4) Reactions of acetyl-coenzyme A.
 (A) GENERATION OF HIGH-ENERGY PHOSPHATE BONDS. The energy of CoA formed by any mechanism can be used to generate ATP from ADP and inorganic phosphate by the reactions shown in 41.
- (B) DONATION OF ACETYL GROUPS. As important as the formation of high-energy phosphate is the role of acetyl-CoA as a form of "active acetate" used directly for synthetic purposes. This function is illustrated in the following two examples.

2 acetyl-CoA

⇒ acetoacetyl-CoA + CoA

40. OH O CH₃ C=O C=O C=O C=O C=O C=O CH₃ CH₃ CH₃
$$\alpha$$
-acetolactic acid α -acetolactic acid

41.

O
$$CH_3$$
— C — S — COA + H_3PO_4 \rightleftharpoons COA + CH_3 — C — O — P — OH
 $acetyl$ - COA

OH
 $acetyl$ - $phosphate$

The formation of acetoacetyl-CoA is probably the first step in the formation of long-chain fatty acids and of the various end products of the butyric acid fermentations (see below).

acetyl-CoA + oxalacetic acid → citric acid + CoA

This reaction is the first step in the complete oxidation of carbohydrates by way of the Krebs cycle (see below).

(C) FORMATION OF OTHER ACYL-COA COMPOUNDS. Coenzyme A serves a function in the metabolism of other metabolically important acids similar to that just described for acetic acid. Acetyl-CoA may react with these acids to form the corresponding acyl-CoA derivatives in reactions catalyzed by the enzyme CoA transphorase. For example,

acetyl-CoA + succinic acid ⇒
acetic acid + succinyl-CoA

The carbon skeletons of almost all cell constituents appear to be synthesized wholly or in part by addition of 2-carbon units to existing carbon chains by the acetylating action of acetyl-CoA. It is therefore apparent that the formation of acetyl-CoA represents a mechanism for the

conservation and transfer of metabolic energy as important as the formation of ATP.

The chief reactions of pyruvic acid in bacteria, a few of which are still to be discussed in later sections of the chapter, are summarized in 42.

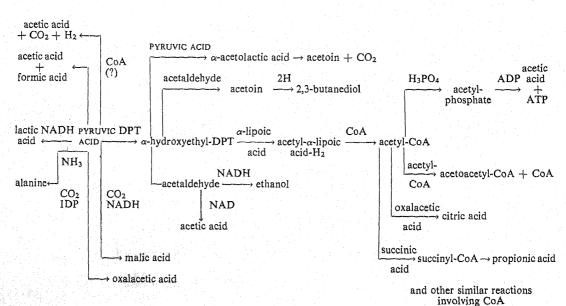
THE TRICARBOXYLIC ACID CYCLE^{21, 22, 24}

Aerobic bacteria can oxidize glucose completely to CO_2 and H_2O with molecular oxygen as the ultimate acceptor of electrons. Glucose and other carbohydrates are converted to pyruvic acid by the different mechanisms already described. The pyruvic acid is then completely oxidized by the concerted action of a series of oxidizing and decarboxylating enzymes operating as a cyclic process. This is the Krebs tricarboxylic acid cycle.

Pyruvic acid enters the cycle by being converted into acetyl-CoA, which in turn condenses with oxalacetic acid to form citric acid (see 43). The 6-carbon citric acid molecule is then decarboxylated and oxidized in a series of reactions to regenerate

42.

Metabolism of Pyruvic Acid in Bacteria



the 4-carbon oxalacetic acid molecule. Thus, the tricarboxylic and dicarboxylic acids of the cycle are not used up; *i.e.*, they act catalytically, but the pyruvic acid molecule is completely oxidized to CO₂ and H₂O. The reader can convince himself of this by summing up the oxygen used and the CO₂ and H₂O formed. (They are shown in boldface type in the diagram. Remember that H₂O is both formed and used up in the Krebs cycle.)

The enzymes involved in the Krebs cycle have been studied in great detail. The enzyme responsible for citric acid synthesis is known as citrate synthetase. Isocitric and malic dehydrogenases are NADP- and NAD-proteins, respectively. The α-ketoglutaric acid oxidase system is very similar to the pyruvic oxidase. Succinic dehydrogenase is a cytochrome-linked dehydrogenase. Oxalosuccinic decarboxylase catalyzes the reversible nonoxidative decarboxylation of oxalosuccinic acid. Two hydrases are also active in the cycle, aconitase and fumarase.

Anything capable of generating acetyl-CoA can be oxidized by way of the Krebs cycle—pyruvic acid, acetic acid, fatty acids, etc. In addition, α -ketoglutaric and oxalacetic acids are in metabolic equilibrium with the key amino acids, glutamic and aspartic acids, respectively. Thus, the Krebs cycle is of great importance, not only as a mechanism of terminal respiration, but also as a common meeting ground for carbohydrate, fat and protein metabolism (see below).

Utilization of the energy liberated in the tricarboxylic acid cycle. The most important product of the complete aerobic oxidation of carbohydrate is not CO₂ or H₂O, but energy. Approximately 10 times as much energy of potential metabolic use is liberated in the complete oxidation of glucose to CO₂ and H₂O as in the glycolysis to lactic acid. While there is a net synthesis of only 2 moles of ATP per mole of glucose converted to lactic acid, about 30 moles of ATP are formed for every mole of glucose oxidized by way of the Krebs cycle. The familiar observation that a facultative anaerobe (such as E. coli), given the same amount of energy source to oxidize, will grow to a much higher cell density aerobically than anaerobically indicates that bacteria derive much more

energy from aerobic oxidations than from fermentations.

The production of ATP via the tricarboxylic acid cycle is regulated by the concentrations of ATP, ADP, and adenosine monophosphate (AMP) in the intact bacterial cell:¹

$$\stackrel{\sim}{ATP} \stackrel{\sim}{=} \stackrel{\sim}{ADP} \stackrel{\sim}{=} \stackrel{AMP}{AMP}$$

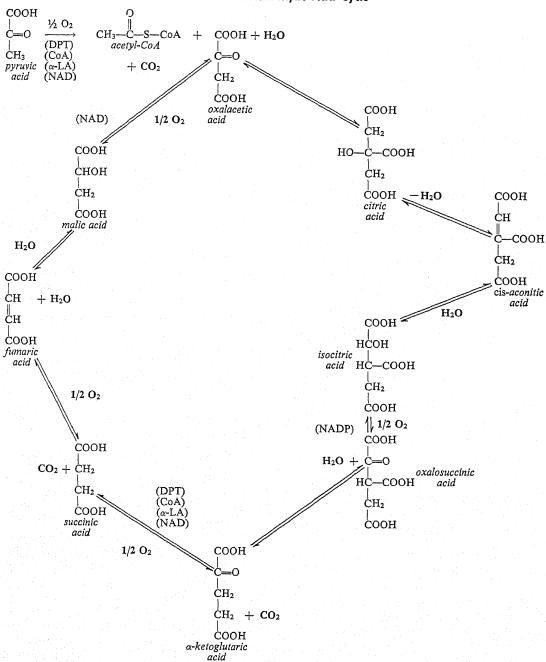
This regulation is achieved by action on three key enzymes, phosphofructokinase of the Embden-Meyerhof scheme (see 24) and citrate synthetase and isocitric dehydrogenase of the tricarboxylic acid cycle (see 43). AMP acts as a positive effector to combine with these enzymes at sites different from those binding their substrates (allosteric sites) in such a way that their rates of enzymatic activity are increased and thus also the rate of ATP synthesis. Since the concentration of AMP varies inversely with that of ATP, an increase in ATP concentration in the cell will reduce the rate of its synthesis, an example of negative feedback (or end product) control. There are probably also as vet undescribed mechanisms for regulating the flow of intermediates and reduced pyridine nucleotides through the tricarboxylic acid cycle and closely associated pathways.

The tricarboxylic acid cycle as a source of intermediates. The tricarboxylic acid cycle also serves an important synthetic function by providing carbon skeletons for the biosynthesis of many important cell constituents as, for example, α -ketoglutaric acid for the synthesis of glutamic acid. However, since each member of the cycle functions catalytically—one molecule is used up and one is formed for each turn of the cycle—this tapping of the tricarboxylic acid cycle for carbon skeletons will bring the operation of the cycle to an immediate halt if the acids withdrawn are not replaced.

Replacement occurs at the level of the 4-carbon dicarboxylic acids either by the fixation of carbon dioxide into 3-carbon acids (see below) or by a modification of the tricarboxylic acid cycle, the glyoxylic acid cycle (45). The two reactions of the glyoxylic acid cycle not shared with the conventional tricarboxylic acid cycle are catalyzed by the enzymes isocitratase and

43.

The Krebs Tricarboxylic Acid Cycle



Net reaction: $C_3H_4O_3 + 2 \frac{1}{2} O_2 \rightarrow 3 CO_2 + 2 H_2O$ pyruvic
acid

malate synthetase (see 44). Diagram 45 shows that for each turn of this modified cycle, two acetic acid molecules are converted into one molecule of succinic acid.

Since the tricarboxylic acid cycle generates energy and no intermediates, while the glyoxylic, acid cycle generates intermediates but no energy, the bacterial cell must regulate the amount of acetyl-CoA fed into each cycle in order to meet changing metabolic demands. Regulation is achieved by control of both the activity and the synthesis of isocitratase. This enzyme is strongly inhibited by succinic acid, one of the products of its action. Thus, it is active when the succinic acid concentration is low and inactive when it is high. Most bacteria synthesize some isocitratase under all conditions, but its production is inhibited when succinic acid is added to the growth medium and

stimulated when acetic acid is the sole source of carbon. When acetic acid and other 2-carbon compounds are the sole sources of carbon, they must be assimilated by way of the glyoxylic acid cycle. Therefore, it must function at a high rate under these conditions.

FIXATION OF CARBON DIOXIDE

Almost all organisms possess, in widely varying degree, the ability to fix CO₂ into organic linkage. The general requirements for fixation are a source of energy and a source of electrons, because the reaction is an energy-requiring reduction. Living cells meet these requirements in many ways, but two main kinds of CO₂ fixation are

45.

The Glyoxylic Acid Cycle

Net reaction:

clearly distinguished: autotrophic fixation and heterotrophic fixation.

Autotrophic and heterotrophic ways of life. Introduction of these terms demands definition and explanation. All parasitic or saprophytic microorganisms derive their energy and most of their carbon from the oxidation of preformed organic compounds. In terms of the classification to be presented shortly, they are *chemoheterotrophs*. There is another large and widely distributed group of microorganisms of great biochemical interest and of critical importance in soil fertility and in the cycles of carbon, nitrogen, and sulfur in nature. These are the autotrophic bacteria. They obtain their energy from inorganic compounds or from light and their carbon from CO₂.

The word "autotrophic" means simply "self-nourishing." In the sense used here, this refers to the lack of any *organic* nutritional requirement. Autotrophic bacteria obviously have the same general nutritional needs as heterotrophs: sources of energy, carbon, nitrogen, and essential inorganic ions

It is appropriate at this point to inquire what is the essential difference between an autotroph and a heterotroph. Consider a representative of each metabolic type: the heterotroph E. coli grows in a medium containing only inorganic salts, ammonia, and an organic compound such as glucose; the autotroph Thiobacillus thiooxidans grows in a medium containing ammonia, elementary sulfur, and inorganic salts. The one absolute difference between the two is this: E. coli derives all of its energy from glucose oxidation and must have this oxidizable organic compound as an energy source in order to grow, while Thiobacillus thiooxidans oxidizes sulfur for its energy and is completely independent of any organic energy supply. The second difference between E. coli and Thiobacillus thiooxidans is that the autotroph uses CO₂ as its sole carbon source and from CO₂ synthesizes all the organic carbon of its cell. Now, while E. coli can also utilize CO₂, it is insufficient as a sole carbon source, and preformed organic carbon is also necessary for growth. Therefore, the second difference, the utilization of CO₂, is not absolute, but a matter of degree.

These differences in energy-yielding

mechanisms and in the extent to which CO₂ serves as a carbon source are probably the only differences between autotrophs and heterotrophs. In every other respect, the metabolism of autotrophic organisms appears to be just the same as that of heterotrophic organisms.

A useful classification of metabolic types of microorganisms based on energy and carbon requirements is as follows:

METABOLIC TYPE Autotroph	ENERGY SOURCE	ORGANIC CARBON REQUIRED	
chemo-autotroph	Inorganic com- pounds	No	
photo-autotroph	Light	No	
Heterotroph			
chemoheterotroph	Organic compounds	Yes	
photoheterotroph	Light	Yes	

All types except the photoheterotrophs will be discussed in this section. Note that not all photosynthetic bacteria are also autotrophic. One point to be remembered in using this classification is that a given organism may, under different conditions, show different types of metabolism. Taking two examples, Hydrogenomonas can live as a chemoautotroph by oxidizing hydrogen or as a chemoheterotroph by oxidizing organic compounds. It is said to be a facultative autotroph. Such behavior is common; there are only a few obligate autotrophs. Similarly, Rhodospirillum rubrum grows anaerobically in the light as a photoheterotroph and aerobically in the dark as a chemoheterotroph.

Autotrophic fixation of carbon dioxide.⁶ The cyclic mechanism whereby CO₂ is fixed into organic linkage by autotrophic organisms was discovered by Calvin and his associates in their studies on photosynthesis in unicellular green algae. However, the same cycle has also been shown to operate in the fixation of CO₂ by photoautotrophic and chemo-autotrophic bacteria. Therefore, the original designation of the cycle as "the photosynthetic carbon reduction cycle" may be broadened to "the autotrophic carbon reduction cycle."

Only two enzymes are unique to this cycle. The rest also function in other areas of carbohydrate metabolism. One of the new enzymes is *phosphoribulokinase*, which

catalyzes the reaction shown in 46. The other enzyme, carboxydismutase, catalyzes the actual fixation reaction in which CO₂ is fixed to ribulose-1,5-diphosphate with the formation of two molecules of 3-phosphoglyceric acid. The exact reaction mechanism is still in doubt, but it is usually formulated as in 47.

The remaining reactions of the autotrophic carbon reduction cycle are concerned with (1) the reduction of 3-phosphoglyceric acid to 3-phosphoglyceraldehyde and (2) the regeneration of ribulose-5-phosphate. The reactions of the cycle are given in detail

Reaction c through g also occur in Embden-Meyerhof glycolysis, while reactions h through m are also a portion of the pentose cycle.

The autotrophic carbon dioxide fixation cycle may be regarded as a modified pentose cycle in which occurs the unique reaction catalyzed by carboxydismutase. It has already been pointed out that CO2 fixation is an energy-requiring reduction. It is now apparent that the energy is supplied by ATP

46.

$$\begin{array}{cccc} CH_2OH & CH_2OPO_3H_2 \\ \hline C=&& C=&O \\ \hline +COH & +ATP & \rightarrow & HCOH & +ADP \\ \hline +COH & +COH & +COH \\ \hline -CH_2OPO_3H_2 & CH_2OPO_3H_2 \\ \hline ribulose-5-P & ribulose-1, 5-diP \\ \end{array}$$

47.

48.

```
(a) 3 ribulose-5-P + 3 ATP \rightarrow 3 ribulose-1,5-diP + 3 ADP
(a) 3 HDUIOSE-3-F + 3 A I F → 3 HDUIOSE-1,3-dIF + 3 ADF

(b) 3 ribulose-1,5-diP + 3 CO<sub>2</sub> + 3 H<sub>2</sub>O ⇒ 6 3-P-glyceric acid

(c) 6 3-P-glyceric acid + 6 ATP → 6 2,3-diP-glyceric acid + 6 ADP

(d) 6 2,3-diP-glyceric acid + 6H+ + 6(NADH or NADPH) ⇒ 6 3-P-glyceraldehyde + 6(NAD+ or NADP+) + 6 H<sub>3</sub>PO<sub>4</sub>

(e) 2 3-P-glyceraldehyde ⇒ 2 dihydroxyacetone-P

(f) 3-P-glyceraldehyde + dihydroxyacetone-P ⇒ fructose-1,6-diP

(g) fructose-1,6-diP + H<sub>2</sub>O → fructose-6-P + H<sub>3</sub>PO<sub>4</sub>

(h) fructose-6-P → DPT → graphene 4-P → dihydroxyathyl DPT
(h) fructose-6-P + DPT = erythrose-4-P + dihydroxyethyl-DPT
(l) erythrose-4-P + dihydroxyacetone-P = sedoheptulose-1,7-diP
 (j) sedoheptulose-1,7-diP + H_2O \rightarrow sedoheptulose-7-P + H_3PO_4 (k) sedoheptulose-7-P + DPT \rightleftharpoons xylulose-5-P + dihydroxyethyl-DPT
 (1) xylulose-5-P = ribulose-5-P
 (m) 2 3-P-glyceraldehyde + 2 dihydroxyethyl-DPT \rightleftharpoons 2 ribulose-5-P + DPT
```

Net reaction: $3 \text{ CO}_2 + 9 \text{ ATP} + 6\text{H}^+ + 6\text{(NADH or NADPH)} + 5 \text{ H}_2\text{O} \rightarrow 3\text{-P-glyceraldehyde} + 9 \text{ ADP} +$ 6(NAD+ or NADP+) + 8 H₃PO₄ and the reducing power by reduced pyridine nucleotide.

Carbon dioxide fixation by chemo-auto-trophic bacteria. These organisms generate the ATP and reduced pyridine nucleotide required for CO₂ fixation by oxidizing inorganic compounds. They can grow in the dark in the presence of only CO₂, inorganic nitrogen, and essential inorganic ions. Although chemo-autotrophs have no organic requirements, they usually have very specific requirements as to energy source. Many can live as autotrophs only by oxidizing a single inorganic substrate. The principal chemo-autotrophic bacteria and their energy-yielding reactions are given in the table below:

Carbon dioxide fixation by photo-autotrophic bacteria.³⁷ Green plants generate their ATP and reduced pyridine nucleotide for CO₂ fixation from the energy of light in reactions occurring in the chloroplasts.

(a) ADP + P
$$\xrightarrow{\text{light}}$$
 ATP

(b) ADP + P + NADP+ +
$$H_2O \xrightarrow{\text{light}} ATP + NADPH + H^+ + \frac{1}{2}O_2$$

Reaction a, termed cyclic photophosphorylation, also occurs in photosynthetic bacteria, but reaction b does not. In b, NADP is reduced by way of the photochemical cleavage of H_2O , a reaction peculiar to green plant photosynthesis. In

the photo-autotrophic green and purple bacteria, an oxidizable inorganic substrate is substituted for H_2O , and reduced pyridine nucleotide is generated enzymatically in the absence of a photochemical reaction. The most commonly employed substrate is H_2S .

Heterotrophic fixation of CO_2 . CO_2 may be fixed by heterotrophs in $C_1 + C_1$, $C_1 + C_2$, $C_1 + C_3$ additions, etc. Of these, the $C_1 + C_3$ reactions are probably of greatest quantitative importance and will be the only ones discussed here. For other important modes of CO_2 fixation, see 57.

Carboxylation of pyruvic acid to malic acid. The malic enzyme which catalyzes this reaction is widely distributed in bacteria.

pyruvic acid +
$$CO_2$$
 + $NADPH$ + $H^+ \longrightarrow malic$ acid + $NADP^+$

Carboxylation of pyruvic acid to oxalacetic acid. This reaction occurs in at least two different ways. Phosphoenolpyruvate is an intermediate in both. In one reaction, its phosphate group is transferred to a nucleotide diphosphate, and in the other it is released as inorganic phosphate.

(a) phosphoenolpyruvic acid + CO_2 + IDP (or GDP or ADP) \rightleftharpoons oxalacetic acid + ITP (or GTP or ATP) (b) phosphoenolpyruvic acid + $CO_2 \rightarrow$

oxalacetic acid + P

Chemo-autotrophic Bacteria

GROUP	TYPICAL REPRESENTATIVE	ENERGY-YIELDING OXIDATION	AEROBIC OR ANAEROBIC	OBLIGATE AUTOTROPH
Sulfur bacteria	Beggiatoa	ΓH ₂ S→S	Aerobic	No
		LS→SO ₄		
	Thiobacillus thioparus	$S_2O_3 \rightarrow SO_4 + S$	Aerobic	Yes
	Thiobacillus thiooxidans	S→SO ₄	Aerobic	Yes
	Thiobacillus denitrificans	S→SO ₄	Anaerobic	Yes
	Thiobacillus ferrooxidans	Fe ⁺⁺ →Fe ⁺⁺⁺	Aerobic	Yes
Nitrifying bacteria	Nitrosomonas)	$NH_3 \rightarrow NO_2$	Aerobic	Yes
	Nitrosococcus			
	Nitrobacter	$NO_2 \rightarrow NO_3$	Aerobic	Yes
Iron bacteria Gallionella Leptothrix	Gallionella	Fe++→Fe+++	Aerobic	Yes (?)
	Leptothrix	Fe++→Fe+++	Aerobic	No
		¹ / ₂ O ₂		
Hydrogen bacteria	Hydrogenomonas	$H_2 \longrightarrow H_2O$	Aerobic	No
		SO₄ To the second seco		
	Desulfovibrio desulfuricans	$H_2 \xrightarrow{5004} H_2 S$	Anaerobic	No
	보면 회장 가는 그 보고 하는 그 회장 그는 것이다. 그는 항상 경상에 가지 않는 것이다.	CO ₂		
	Methanobacterium omelianskii	$H_2 \longrightarrow CH_4$	Anaerobic	No

Carboxylation of propionic acid to succinic acid. This mode of CO₂ fixation is found chiefly in the propionic acid bacteria. It occurs as shown in 49.

The importance of CO₂ fixation. Organic carbon is constantly being converted into CO₂ by the respiratory activity of all living cells and by the breakdown of dead organic matter by saprophytic microorganisms. The CO₂ fixation of green plant photosynthesis is largely responsible for the return of this CO₂ to organic forms available to nonphotosynthetic organisms. Turning from the role of CO₂ in the economy of nature as a whole to its role in the economy of individual organisms, it is evident that, by definition, autotrophic organisms are completely dependent upon CO₂ as a carbon source. However, heterotrophic organisms cannot grow in the complete absence of CO₂, so CO₂ fixation must play a vital role in their metabolism as well. It is highly probable that at least one of the essential functions of CO₂ in heterotrophic economy is the replenishment of the 4-carbon members of the tricarboxylic acic cycle by means of the $C_1 + C_3$ additions just discussed.

BACTERIAL FERMENTATIONS⁴²

Bacteria anaerobically decompose, or *ferment*, carbohydrates to a relatively small number of 1-, 2-, 3-, and 4-carbon compounds. As indicated in 50, fermentation end products are usually produced by the breakdown of glucose to pyruvic acid. The pyruvic acid then reacts in various ways, all of which have already been discussed, to form the typical end product of fermentation (indicated by boldface type in 50). In a general way, fermentation end products may be regarded as the reduction products of fermentation with CO₂ as the oxidation product.

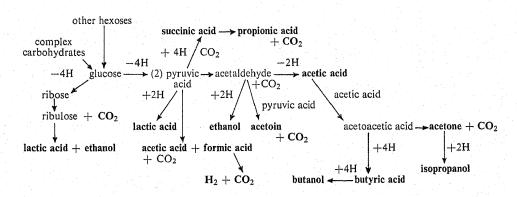
The chief metabolic value of the anaerobic breakdown of carbohydrate beyond the pyruvic acid stage lies in the extra energy

49.

$$\begin{array}{c} \text{CH}_3\text{CH}_2\text{COOH} + \text{CoA} & \longrightarrow \text{CH}_3\text{CH}_2\text{C} - \text{S} - \text{CoA} \\ \textit{propionic acid} & \textit{propionyl-CoA} \\ \text{O} & \text{O} & \text{O} \\ \text{CH}_3\text{CH}_2\text{C} - \text{S} - \text{CoA} + \text{CO}_2 & \xrightarrow{\text{biotin}} \text{CH}_3\text{CHC} - \text{S} - \text{CoA} \\ & \text{COOH} \\ \textit{methyl malonyl-CoA} \\ \text{O} & \text{O} \\ \text{CH}_3\text{CHC} - \text{S} - \text{CoA} & \xrightarrow{\text{E}_{12}} \text{CH}_2\text{CH}_2\text{C} - \text{S} - \text{CoA} \\ & \text{COOH} \\ \textit{succinyl-CoA} \\ \end{array}$$

50.

Bacterial Fermentation of Carbohydrates



made available, usually in the form of ATP. Bacterial fermentations are of practical importance because they produce products of industrial value (acetone, butanol, and so on) and because species differences in fermentation patterns are of great value in identifying and differentiating bacteria.

Most of the essential mechanisms involved in the metabolic transformations of bacterial fermentation have been considered in previous sections. The purpose of this section is to describe a few of the main types of fermentation patterns exhibited by important groups of bacteria.

Alcoholic fermentation. The oldest known type of fermentation is the production of ethanol from glucose.

Yeasts carry out an almost pure alcoholic fermentation, but although some alcohol is produced by many bacteria, it is the major end product in only a relatively few species.

Glucose is converted by way of the Embden-Meyerhof pathway to pyruvic acid, which is then decarboxylated to acetaldehyde by pyruvic carboxylase. The acetaldehyde is reduced to ethanol by alcohol dehydrogenase. NADH is the cofactor.

Lactic acid fermentation. In a typical lactic fermentation, pyruvic acid produced from glucose by way of the Embden-Meyerhof scheme is reduced by NADH formed in the oxidation of 3-phosphogly-ceraldehyde.

pyruvic acid + NADH + $H^+ \rightarrow$ lactic acid + NAD+

A very large number of bacteria form some

lactic acid, and it is the major fermentation end product of many (Lactobacillus, Streptococcus, Leuconostoc, Bacillus). If a given species, such as L. casei, produces only lactic acid from glucose by way of the Embden-Meyerhof pathway, it is said to possess a homolactic fermentation or to be a homofermenter. Others, such as Lactobacillus brevis, produce large quantities of lactic acid, but in addition form other end products such as acetic acid, ethanol, and CO2. They are called heterofermenters, and the fermentation is termed heterolactic (see below). A single species may show both types of lactic fermentation, depending upon such environmental factors as pH and substrate.

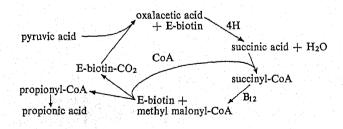
In heterolactic fermentations it was formerly assumed that the end products are formed by way of the Embden-Meyerhof scheme with pyruvic acid as an intermediate. However, this is not always true. As has already been seen in previous sections, glucose may be broken down by way of pentose or the Entner-Doudoroff pathway to typical heterofermentative end products such as CO₂, ethanol, lactic acid, and acetic acid without the participation of phosphorylating glycolysis.

Propionic acid fermentation. The propionic acid bacteria are closely related to the lactobacilli and get their name from their characteristic production of propionic acid from glucose. The propionic acid bacteria also form acetic and succinic acids and CO₂ as typical fermentation end products.

Propionic acid is formed from pyruvic acid by the cyclic process shown in 51. The cycle

51.

Formation of Propionic Acid



Net reaction: pyruvic acid + 4H → propionic acid + H₂O involves two novel reactions which merit attention (see 52).

First, CO₂ is transferred from methyl malonyl-CoA to pyruvic acid by a biotin-containing transcarboxylase without the formation of free CO₂. Biotin is also involved in other reactions of transcarboxylation.

Second, the conversion of succinyl-CoA to methyl malonyl-CoA requires the transformation of a straight-chain compound into a branched chain one. This is brought about by an isomerase containing a coenzyme form of vitamin B_{12} (methyl- B_{12}) (53). Similar "chain-straightening" reactions also occur in the metabolism of amino acids.

Fermentations of the coliform group of bacteria. Coliform bacteria ferment glucose to a variety of end products. E. coli produces lactic acid and ethanol by lactic and alcoholic fermentations. Lactic acid, acetic acid, and CO₂ are formed by the dismutation of two moles of pyruvic acid. By dismutation is meant the oxidation of one molecule and the reduction of the other. One molecule of pyruvic acid is oxidized to acetic acid and CO₂ as already described.

 $CO_2 + NADH + H^+$

The other is reduced to lactic acid by lactic dehydrogenase and the NADH produced in the oxidation of the first molecule of pyruvic acid.

pyruvic acid + NADH + H → lactic acid + NAD+

The net reaction is:

2 pyruvic acid → acetic acid + lactic acid + CO₂

Acetic and formic acids are also formed by a reaction restricted to the enteric bacteria, the *phosphoroclastic* reaction. The overall reaction is:

pyruvic acid + H₃PO₄ = acetyl-P + formic acid

This reaction is CoA and DPT dependent:

pyruvic acid + CoA
$$\Longrightarrow$$
 acetyl-CoA + formic acid acetyl-CoA + H₃PO₄ \Longrightarrow acetyl-P + CoA

ATP may be generated from acetylphosphate by reactions already described. If *E. coli* is grown in a medium with ammonium salts as the nitrogen source, formic acid is not further attacked, but if the medium contains certain amino acids, the enzyme *hydrogenlyase* is formed which breaks down formic acid to CO₂ and H₂.

The fermentation of glucose by Aerobacter aerogenes is similar to that of E. coli except that a considerable fraction of the fermented glucose is converted to acetoin. The production of acetoin is the basis for both the methyl red and the Voges-Proskauer tests for differentiation of E. coli from the aerogenes (cloaca) type of coliform bacilli. The Voges-Proskauer test is a color test for acetoin, while the formation of the neutral acetoin instead of acetic and formic acid makes the cloaca culture less acidic and thus methyl red-negative.

Butyl alcohol fermentations. Anaerobic organisms such as Cl. butylicum, Cl. aceto-butylicum, Cl. welchii, and Cl. kluyveri

52.

$$\begin{array}{c} transcarboxylase \\ methyl \ malonyl\text{-CoA} + E\text{-biotin} \xrightarrow{\hspace*{1cm}} propionyl\text{-CoA} + E\text{-biotin-CO}_2 \\ E\text{-biotin-CO}_2 + pyruvic \ acid \rightarrow oxalacetic \ acid + E\text{-biotin} \end{array}$$

53.

COOH

$$CH_2$$
 EH_2
 EH_2

possess glucose fermentations characterized by the accumulation of butanol, butyric acid, and related products. The key reaction in the formation of such compounds is the CoA-catalyzed formation of acetoacetic acid. From acetoacetic acid are formed the end products typical of the butyl alcohol fermentation (see 54).

Lipid Metabolism

Bacteria contain a variety of lipids, the kind and amount frequently being highly dependent upon the exact conditions of culture. These lipids may be specifically incorporated into subcellular structures, such as cell walls and cytoplasmic membranes, or they may be stored as food reserves.

Hydrolysis of lipids.¹⁸ Neutral fats, which consist of a molecule of glycerol esterified with three fatty acid molecules, are hydrolyzed by lipases.

Phosphatides, or phospholipids, contain a molecule of glycerol, two molecules of fatty acid, a molecule of phosphoric acid, and a nitrogen base which may be choline, ethanolamine, or serine.

Choline-containing phosphatides are called *lecithins* (phosphatidylcholine). Those with ethanolamine and serine are called *cephalins* (more specifically, phosphatidylethanolamine and phosphatidylserine).

Lecithin may be used to illustrate the breakdown of phosphatides by bacteria (see 55). Phosphatidase A (lecithinase A) hydrolyzes off one fatty acid molecule to produce lysolecithin, a potent hemolytic agent. Lysolecithinase splits off another fatty acid to form glycerylphosphorylcholine. The same product is formed directly by the

54.

55.

Enzymic Hydrolysis of Lecithin

action of phosphatidase B. Phosphatidase D (lecithinase D) splits lecithin into a diglyceride and phosphorylcholine. This enzyme is identical with the α -toxin of Clostridium welchii.

Oxidation of fatty acids.¹² Fatty acids are oxidized by a mechanism closely similar to, but not identical with, the mitochondrial system for oxidation of fatty acids in higher animals. With butyric acid as an example, fatty acid oxidation proceeds in *Cl. kluyveri* as shown in 56. Reactions (2) through (5) are the same as in animal tissues, while reactions (1) and (6) are peculiar to bacteria.

Synthesis of fatty acids. 19 Bacteria synthesize saturated fatty acids with even

(1) $CH_3COSC_0A + ACP-SH =$

numbers of carbon atoms by the same pathway as do mammalian cells. The first step is the synthesis of malonyl-CoA from CO₂ and acetyl-CoA by the biotin-containing enzyme acetyl-CoA carboxylase (see 57). Compare these reactions with those in 49. In Cl. kluyveri and E. coli, further steps in fatty acid synthesis require a fatty acid synthetase which has 4'-phosphopantetheine (see 104 for structural formula) as its prosthetic group. This enzyme binds acyl groups as thioesters and has been called the acyl carrier protein (ACP). Six enzymes are required for converting acetyl-CoA and malonyl-CoA to butyric acid (58). Each enzyme requires ACP or the appropriate

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56.
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57.

$$CO_2 + ATP + E$$
-biotin \longrightarrow CO_2 -E-biotin $+ ADP + P$
 CO_2 -E-biotin $+ CH_3COSCoA = E$ -biotin $+ HOOCCH_2COSCoA$
 $malonyl$ - CoA

acetylphosphate

CH₃COS-ACP + CoA-SH

58.

(2) HOOCCH₂COSCoA + ACP-SH
$$\xrightarrow{\text{malonyl}}$$
 HOOCCH₂COS-ACP + CoA-SH $\xrightarrow{\text{transacetylase}}$ HOOCCH₂COS-ACP + CoA-SH $\xrightarrow{\text{transacetylase}}$ CH₃COS-ACP + HOOCCH₂COS-ACP $\xrightarrow{\beta\text{-ketoacyl-ACP}}$ CH₃COCH₂COS-ACP + ACP-SH $\xrightarrow{\beta\text{-ketoacyl-ACP}}$ CH₃COCH₂COS-ACP + NADPH + H+ $\xrightarrow{\beta\text{-ketoacyl-ACP}}$ CH₃CHOHCH₂COS-ACP + NADP+ $\xrightarrow{\beta\text{-ketoacyl-ACP}}$ CH₃CHOHCH₂COS-ACP + NADP+ $\xrightarrow{\beta\text{-ketoacyl-ACP}}$ CH₃CHOHCH₂COS-ACP + NADP+ $\xrightarrow{\beta\text{-ketoacyl-ACP}}$ CH₃CHOHCH₂COS-ACP + NADP+ $\xrightarrow{\alpha\text{-noyl-ACP}}$ CH₃CH=CHCOS-ACP + H₂O $\xrightarrow{\alpha\text{-noyl-ACP}}$ CH₃CH=CHCOS-ACP + NADP+ $\xrightarrow{\alpha\text{-noyl-ACP}}$ C

reductase

(butyryl-)

59.

CH₃ (CH₂)₁₆ COSCoA
$$\xrightarrow{\text{NADP, O}_2}$$
 CH₃ (CH₂)₇ CH=CH (CH₂)₇ COSCoA (stearic acid) (oleic acid)

60.

acyl-ACP. The butyryl-ACP formed in reaction 58 (6) can be split to butyric acid or condensed with another molecule of malonyl-CoA to initiate another cycle of synthesis.

Saturated fatty acids can be oxidatively desaturated to unsaturated acids by enzymes requiring both molecular oxygen and NADP (59). Fatty acids containing cyclopropane rings are frequently found in bacterial lipids. They are synthesized by addition of a methylene group to the corresponding unsaturated fatty acid (60). The role of S-adenosylmethionine in reactions of 1-carbon transfer will be discussed later.

Synthesis of triglycerides and phospholipids. Triglycerides are probably syn-

the sized in bacteria by the esterification of glycerol with appropriate acyl-CoA compounds, but exact information is lacking at this time.

The phospholipids of *E. coli* are made as illustrated in **61**. The appropriate phosphatidic acid is synthesized from glycerol and acyl-CoA. It then reacts with cytidine triphosphate to form cytidine diphosphate diglyceride. The role of cytidine nucleotides in phospholipid synthesis is thus similar to the role of uridine nucleotides in polysaccharide synthesis. Serine is added to the cytidine diphosphate diglyceride to form *phosphatidylserine* which is decarboxylated to *phosphatidylethanolamine*.

Inorganic Nitrogen Metabolism 29

Nitrogen is an essential constituent of the last two great groups of metabolites to be considered, the amino acids and proteins and the nitrogen bases and nucleic acids. The metabolism of inorganic forms of nitrogen presents certain peculiar problems stemming mainly from the unusual unreactivity of molecular nitrogen as compared with that of other elements near it in the periodic table—carbon, oxygen, silicon, phosphorus, and sulfur. These elements occur in large quantities in the rocks of the earth's crust, but nitrogen-containing minerals are much rarer. Thus, the bulk of this vital element has remained as molecular nitrogen in the atmosphere.

Nitrate metabolism. Green plants, the ultimate food source of all nonphotosynthetic organisms, can generally use the nitrates found in all soils and natural waters

as their sole nitrogen sources. These nitrates are mainly obtained, not from nitrate deposits, which are very scarce, but from the breakdown of dead plant and animal matter in the soil.

Ammonia is produced in organic decompositions carried out by a wide variety of microorganisms. However, the oxidation of ammonia to nitrate can be accomplished by only a few specialized obligately aerobic, obligately autotrophic soil microorganisms of universal distribution. The first step is the work of bacteria of the genera Nitrosomonas and Nitrosococcus:

$$2 \text{ NH}_3 + 3 \text{ O}_2 \rightarrow 2 \text{ HNO}_2 + 2 \text{ H}_2\text{O}$$

The nitrite is further oxidized to nitrate by Nitrobacter:

$$2 \text{ HNO}_2 + O_2 \rightarrow 2 \text{ HNO}_3$$

Synthesis of Phosphatidylserine and Phosphatidylethanolamine in Escherichia coli

This process is called *nitrification* and the responsible organisms *nitrifying bacteria*.

Although few organisms can oxidize ammonia to nitrate, many higher plants, fungi, and bacteria can reduce nitrate to ammonia and thus can satisfy their inorganic nitrogen requirements with nitrate. Nitrate is reduced to nitrite by nitrate reductase and then to ammonia by nitrite reductase. These reductases are usually pyridine nucleotide-linked metalloflavoproteins. Ferredoxin may act as an electron carrier in the reduction of nitrite.

The nitrogen cycle in nature may then be represented in a most simplified manner by:

Some Agents of Nitrogen Fixation

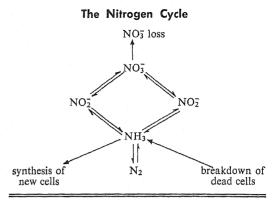
REPRESENTATIVE SPECIES	TYPE OF METABOLISM
Azotobacter chroococcum	Aerobic, heterotrophic
Clostridium pasteurianum	Anaerobic, heterotrophic
Nostoc (blue-green algae)	Aerobic, photosynthetic
Rhodospirillum rubrum	Anaerobic, photosynthetic
Rhizobium leguminosarum	Aerobic, heterotrophic; fixation occurs only in association with leguminous plants

However, the cycle is not perfect and nitrates are constantly lost to biological use. In terrestrial nitrogen cycles, nitrates are constantly leached away by soil water and eventually lost in the ocean. In addition, some soil bacteria, the *denitrifying bacteria*, reduce nitrate to molecular nitrogen.

Nitrogen fixation.³⁰ If there were unlimited amounts of soil nitrate, this constant loss would be of no importance. However, since this is not so, it is fortunate that a number of microorganisms can reduce molecular nitrogen to ammonia and thus make up the deficit. Nitrogen fixation is a widespread but sporadically occurring metabolic process. While comparatively few microorganisms have been positively identified as nitrogen fixers, the organisms known to be capable of reducing molecular nitrogen include a wide variety of metabolic types as seen in the accompanying table.

The first definitely identified product of the reduction of molecular nitrogen is ammonia. Some success has been achieved in understanding the mechanism of nitrogen fixation in one organism, the anaerobic nitrogen-fixer Cl. pasteurianum. Cell-free extracts from this bacterium loosely couple the oxidation of pyruvate with the reduction of molecular nitrogen to ammonia. Ferredoxin is the electron carrier that couples these two systems. About 100 moles of pyruvate must be oxidized to bring about the reduction of 1 mole of nitrogen. Since ferredoxin does not occur in aerobes such as Azotobacter, these nitrogen-fixers must

62.



use other mechanisms for reducing nitrogen to ammonia.

The previous nitrogen cycle may now be enlarged to include nitrate loss and its replacement by nitrogen fixation, as shown in 62

Primary uptake of ammonia into organic linkage. Since the nitrogen in most organic compounds of biological importance is at the state of oxidation of ammonia, it is not surprising that microorganisms generally introduce nitrogen into organic linkage in the form of ammonia.

Four main pathways for the primary uptake of ammonia are outlined in 63, together with examples of important nitrogen-containing metabolites synthesized by each pathway. The detailed mechanisms of these reactions will be given at appropriate places in succeeding sections.

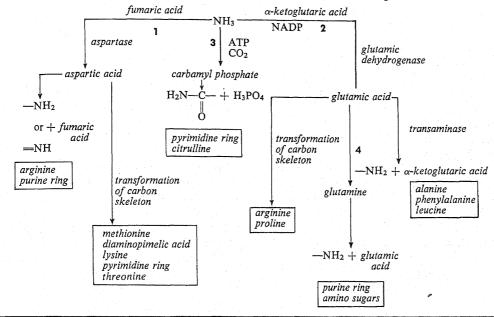
Metabolism of Amino Acids and Proteins²⁸

Amino acids occur in bacteria in the free state, in peptides, and in proteins. Most of them are found in proteins, but the small quantity of amino acids in the free state and in peptides is of great metabolic importance. Amino acids are not important energy sources for bacteria, except for the proteolytic anaerobes. Nevertheless, many bacteria degrade amino acids as well as incorporate them into protein. Both the degradative and synthetic phases of amino acid metabolism are closely integrated with the metabolism of carbohydrates, particularly through the reactions of the tricarboxylic acid-glyoxylic acid cycles.

BREAKDOWN OF PROTEINS35

Intact protein molecules do not readily pass into the bacteria cell and must be hydrolyzed to smaller units by extracellular enzymes before they can be utilized as nitrogen sources. Relatively few bacteria possess such extracellular proteolytic enzymes. Bacteria are usually separated into fermentative and proteolytic types according to whether carbohydrate oxidation or proteolysis characterizes their biochemical activities. Cl. sporogenes, Cl. histolyticum, Proteus vulgaris, and Ps. aeruginosa are examples of actively proteolytic bacteria.

Primary Uptake of Ammonia into Organic Linkage



Bacterial enzymes attack a wide variety of proteins and peptides.

BREAKDOWN OF AMINO ACIDS4

The ability to attack amino acids is much more widespread among bacteria than the ability to attack proteins. Bacteria may be divided into two groups on the basis of the variety of amino acids they are capable of breaking down. Gram-negative organisms such as E. coli, Pr. vulgaris, and Ps. aeruginosa attack almost all of the naturally occurring amino acids. Such organisms are also versatile synthesizers of amino acids and can grow on ammonia and a simple carbon source. In contrast, gram-positive organisms, such as the lactobacilli and the streptococci, have only a very limited ability to catabolize amino acids and also require a large number of amino acids as growth factors. Bacteria, particularly gram-positive species, can accumulate high intracellular concentrations of free amino acids, much higher than the extracellular concentration of the amino acids in the medium. This is accomplished by means of an energy requiring enzyme-like transport mechanism located at or near the cell surface.

Bacteria attack the amino acid molecule

in two main ways: decarboxylation or deamination. The enzymes catalyzing both types of catabolism are usually inducible, appearing only when their substrates are present in the medium.

Decarboxylation of amino acids. Bacteria carry out a nonoxidative decarboxylation of amino acids to the corresponding amines, as shown by the general equation:

$$R-CHNH_2-COOH \rightarrow R-CH_2NH_2+CO_2$$

Each decarboxylase is specific for a single amino acid, and the decarboxylation is complete and irreversible. Enzymes decarboxylating diaminopimelic acid, lysine, arginine, ornithine, histidine, tyrosine, phenylalanine, aspartic acid, and glutamic acid have been described.

With the possible exception of histidine decarboxylase, the amino acid decarboxylases have a common coenzyme, pyridoxal phosphate, which is a derivative of the growth factor pyridoxin or vitamin B₆.

pyridoxal phosphate

Pyridoxal phosphate is also a coenzyme for

other enzymes important in amino acid metabolism (see below).

Deamination of amino acids. Deamination of amino acids by the enteric bacteria. As a group, the enteric bacteria deaminate a large number of amino acids in several different ways.

OXIDATIVE DEAMINATION. Glutamic acid is broken down to ammonia and α -ketoglutaric acid by the glutamic acid dehydrogenase of $E.\ coli$ as shown in 64. This reaction is readily reversible, and glutamic acid can be synthesized from ammonia and α -ketoglutaric acid. Since glutamic acid can transfer its amino group to many other keto acids by transamination (see below), fixation of ammonia nitrogen in glutamic acid by glutamic dehydrogenase is of primary importance in the synthesis of amino acids in general.

Many enteric bacteria (but not E. coli)

contain amino acid oxidases which carry out the aerobic oxidative deamination of amino acids according to the general equation shown in 65. The amino acid oxidases are usually nonspecific with respect to substrate; the oxidase of *Pr. vulgaris* attacks 11 different amino acids.

Nonoxidative deamination. E. coli and related organisms contain enzymes catalyzing the deaminations shown in 66. The deamination of aspartic acid by aspartase is easily reversible and furnishes a second important pathway for the uptake of ammonia into amino acids, since aspartic acid, like glutamic acid, is very active in transamination reactions.

Tryptophan breakdown. E. coli attacks tryptophan only in its side chain and leaves the aromatic rings intact in the form of indole (cf. the degradation of tryptophan by pseudomonads). The reaction is shown in 67. Pyri-

64.

65.

R-CHNH₂-COOH +
$$\frac{1}{2}$$
 O₂ \rightarrow R-CO-COOH + NH₃ amino acid keto acid

66.

67.

doxal phosphate is the coenzyme for the reaction.

In contrast to the limited attack made upon tryptophan by E. coli, most Pseudomonas species attack both the aromatic rings and side chains of the amino acid.

Metabolism of amino acids by obligate anaerobes. The proteolytic species of the genus Clostridium do not ferment carbohydrate and must obtain metabolic energy from the oxidation of amino acids. Since they are obligate anaerobes, the ultimate hydrogen acceptor is not molecular oxygen but the amino acids themselves or their breakdown products.

The clostridia have evolved two methods for the anaerobic oxidation of amino acids. In one, the Stickland reaction, one amino acid is oxidized and one is reduced. In the second, amino acids are decomposed with the liberation of molecular hydrogen.

The Stickland reaction. Cl. sporogenes, Cl. botulinum, and other (but not all) species perform reactions of the general type shown in 68. Amino acid (1) accepts hydrogen atoms from (2) and is reduced to a fatty acid while (2) is oxidized to a keto acid. The equation indicates only the overall course of the reaction. It is likely that at least two enzymes, specific dehydrogenases for the reacting amino acids, and a hydrogencarrying coenzyme, perhaps one of the pyridine nucleotides, are required for a Stickland reaction. The coupled oxidationreduction of amino acids in these reactions proceeds at a rate comparable to the aerobic oxidation of carbohydrate in fermenters, and the Stickland reactions appear entirely capable of serving as the chief energy source for the proteolytic clostridia.

Decomposition of amino acids with liberation of hydrogen. Several clostridia which do not perform Stickland reactions decompose amino acids with liberation of NH₃,

CO₂, and H₂. The mechanism of such an "amino acid fermentation" is unknown.

SYNTHESIS OF AMINO ACIDS4

Heterotrophic bacteria synthesize amino acids from inorganic nitrogen, usually ammonia, and from simple organic carbon compounds such as acetic and pyruvic acids supplied in the medium or derived from the breakdown of other substances, usually carbohydrates. The great variation in structure among the 20-odd amino acids usually found in proteins means that amino acids are made by many different independent synthetic routes. However, several reactions or groups of reactions are of general importance in amino acid synthesis and will be considered before the biosynthesis of individual amino acids is discussed.

Uptake of ammonia. The primary uptake of ammonia into organic linkage has already been discussed (see Inorganic Nitrogen Metabolism). The ability to convert ammonia nitrogen into amino acid nitrogen is a common property of heterotrophic bacteria, and many heterotrophs can grow with ammonia as the sole source of nitrogen.

Mechanisms for the formation of glutamic and aspartic acids from ammonia and the corresponding keto acids have already been given. It is probable that a considerable fraction of the utilized ammonia is incorporated by way of glutamic acid and transamination reactions into many amino acids.

Transamination. Many amino acids undergo the general reaction of transamination (see 69). In transamination, the amino group of one amino acid is transferred to the α -position of an α -keto acid, thus synthesizing a new amino acid and a new keto acid. Transaminases are widely distributed in animals, higher plants, and bacteria.

68.

 R_1 —CHNH₂—COOH + R_2 —CHNH₂—COOH + $H_2O \rightarrow R_1$ —CH₂—COOH + R_2 —COOH + 2 NH₃

The transaminases are yet another group of amino acid enzymes for which pyridoxal phosphate is the coenzyme. Under appropriate conditions another member of the vitamin B₆ family, pyridoxamine phosphate, is also a coenzyme of transamination (see 70). Snell has suggested that these coenzymes function in transaminase systems as amino group carriers. Thus, the reaction between glutamic and oxalacetic acids proceeds as shown in 71.

Although many amino and keto acids participate in transamination reactions, glutamic and α -ketoglutaric acids have been a part of almost all transamination reactions so far described. These two acids may be thought of as an amino-group-carrying system, which may either accept amino groups from other amino acids or donate them to other keto acids. Transaminations are generally reversible reactions, and an amino acid which can donate its amino group to α -ketoglutaric acid can also be synthesized by the reverse reaction from glutamic acid and its keto acid analogue. In bacteria, transamination of the keto acids corresponding to glutamic acid, aspartic acid, alanine, serine, phenylalanine, tyrosine, leucine, isoleucine, and valine appears to be an important synthetic reaction. There are many different transaminases, each with definite substrate specificity.

Racemization and the importance of Damino acids. With the exception of glycine, each α -amino acid has an asymmetric carbon atom and therefore exists as two optical isomers, the D- and L- forms:

The amino acids of proteins are of the Lconfiguration, and it was once thought that only the L- form occurred in nature. The D- forms were often called "unnatural" amino acids, but they are also widely distributed in bacteria. D-Amino acids were first demonstrated in various polypeptides characteristic of the genus Bacillus: in the capsule of B. anthracis (D-glutamic acid) and in the antibiotics gramicidin and tyrocidine from B. brevis (D-leucine, D-valine, and D-phenylalanine). Penicillin contains D-valine and D-cycloserine is a derivative of D-serine. D-Alanine and D-glutamic acid are constituents of the murein component of the bacterial cell wall.

The amino acid racemases are enzymes that catalyze the racemization of optically active amino acids:

Pyridoxal phosphate is the coenzyme for these enzymes. Racemases for alanine, glutamic acid, threonine, methionine, proline, and diaminopimelic acid have been found in a number of bacteria, and it is clear that the ability to interconvert the optical isomers of many amino acids is a widely distributed bacterial property.

Synthesis of individual amino acids. Bio-

70.

71.

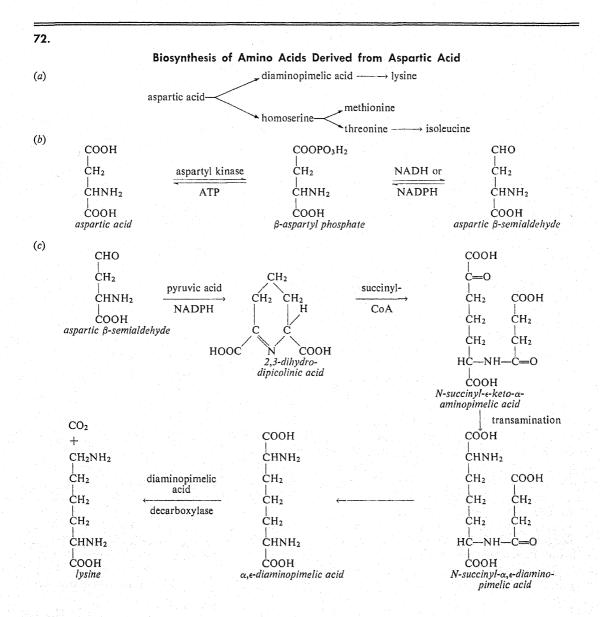
enzyme-CHO + glutamic acid
$$\rightleftharpoons$$
 enzyme-NH₂ + α -ketoglutaric acid (pyridoxal form) (pyridoxamine form) enzyme-NH₂ + oxalacetic acid \rightleftharpoons aspartic acid + enzyme-CHO

synthesis of amino acids derived from aspartic acid. The synthesis of aspartic acid by transamination or by the action of the enzyme aspartase has already been described. Once formed, aspartic acid may serve as the precursor of six other amino acids (see 72a). Of these, homoserine is an important biosynthetic intermediate but is not incorporated into cell structures, diaminopimelic acid is found only as a part of the cell wall muropeptides, and the other four are major constituents of bacterial proteins.

The key intermediate in the further metabolism of aspartic acid is aspartic β -semi-

aldehyde, which is made as shown in 72b. It is then converted into diaminopimelic acid and lysine (72c) or homoserine (72d), which in turn is the precursor of methionine (72d) or of threonine and isoleucine (72e).

The first step in diaminopimelic acid biosynthesis is the condensation of aspartic semialdehyde with pyruvate to yield, after dehydration and reduction, 2,3-dihydro-dipicolinic acid. After reaction with succinyl-CoA and opening of the ring, the second amino group is added by transamination, and the succinyl group is added to yield α, ϵ -diaminopimelic acid. This amino acid is unusual in that it has two asymmetic



72. (cont.) Biosynthesis of Amino Acids Derived from Aspartic Acid (cont.)

carbon atoms and thus exists in D-, L-, and meso-stereoisomers. Diaminopimelic acid racemase catalyzes their interconversion. The L-acid is the immediate synthetic product, but it must be converted into the meso-acid before it can be decarboxylated to lysine. Lysine is also unusual because there are two phylogenetically diverse pathways for its biosynthesis. The pathway in bacteria, higher plants, and some algae and fungi is via diaminopimelic acid. In other algae and fungi, the immediate precursor of lysine is α -aminoadipic acid.

Homoserine is formed from aspartic semialdehyde by reduction of the aldehyde group to a primary alcohol by homoserine dehydrogenase and NADH. It is converted to O-succinyl-homoserine and condensed with cysteine (cysteine biosynthesis will be given later) to yield cystathionine in a reaction catalyzed by cystathionine synthetase. Cystathionine is then cleaved in such a way that the S-atom contributed by cysteine now goes with the carbon skeleton of homoserine to become homocysteine. Methionine is finally formed by addition of a methyl group to the -SH group of homocysteine. An example of the transfer of the 1-carbon unit by S-adenosylmethionine has already been given. Here, the agent of 1-carbon transfer

is an example of the second type of compound involved in these reactions, a coenzyme form of the growth factor folic acid. A coenzyme form of vitamin B_{12} also participates in this reaction. The role of folic acid coenzymes in 1-carbon transfer will also be discussed in a later section of this chapter.

The synthesis of threonine proceeds via homoserine and O-homoserine phosphate which is rearranged to threonine in the presence of a pyridoxal phosphate-dependent enzyme. The branched chain amino acid isoleucine is made by decarboxylation of threonine to α -ketobutyric acid, which is

condensed with pyruvic acid to form the branched chain compound α -aceto- α -hydroxybutyric acid. It is then converted to *isoleucine* via the corresponding dihydroxy acid.

Biosynthesis of valine and leucine. The other branched chain amino acids are valine and leucine. They share the common intermediate α -ketoisovaleric acid, which is made from α -acetolactic acid formed as already described by the condensation of pyruvic acid and α -hydroxyethyl-DPT (36). α -Ketoisovaleric acid may be transaminated to valine (73a) or reacted with acetyl-CoA

to form β -hydroxy- β -carboxy-isocaproic acid which is dehydrated, reduced, and transaminated to *leucine* (73b).

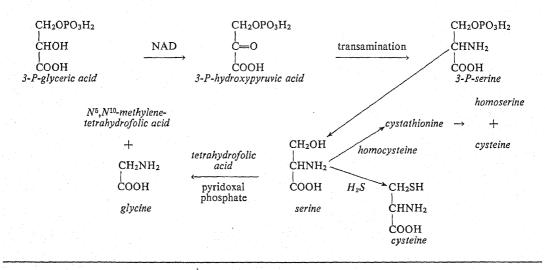
Biosynthesis of serine, glycine, and cysteine. Serine is not only a constituent of bacterial proteins but it is also an intermediate in the synthesis of glycine and cysteine and in the interconversion of methionine and cysteine. Serine is synthesized from the glycolytic intermediate 3-phosphoglyceric acid as shown in 74. It may be converted to glycine in the presence of serine transhydroxymethylase, tetrahydrofolic acid, and pyridoxal phosphate (74). Cysteine may be synthesized directly from serine and H₂S and serine sulfhydrase or indirectly through the intermediation of cystathionine (74).

Biosynthesis of alanine. Alanine may be made in a number of ways (75): transamination of pyruvic acid, reductive amination of pyruvic acid, or the β -decarboxylation of aspartic acid.

Biosynthesis of histidine. Histidine and the purines both have imidazole rings, but they are synthesized by entirely different mechanisms (compare 76 and 85). As shown in 76a, the imidazole ring of histidine contains carbon and nitrogen atoms from 5-phospho- α -ribosyl pyrophosphate, ATP, and glutamine. The first intermediate in histidine biosynthesis to contain an imidazole ring is imidazole glycerol phosphate. It is converted into histidine by the reactions given in 76b.

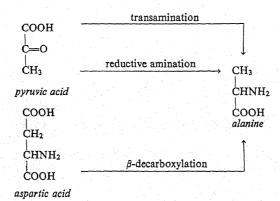
74.

Biosynthesis of Serine, Glycine, and Cysteine



75.

Biosynthesis of Alanine



76.

Biosynthesis of Histidine

Biosynthesis of arginine and proline. These two amino acids are synthesized from glutamic acid through the common intermediate glutamic acid γ -semialdehyde (77a). Proline is made as shown in 77b.

Ornithine, a key intermediate in the synthesis of arginine and a constitutent of some

bacterial cell walls, may be formed by transamination of glutamic γ -semialdehyde (77c) or by a more indirect route involving formation and transamination of N-acetyl-glutamic γ -semialdehyde. Ornithine reacts with carbamyl phosphate synthesized as indicated by the equation in 77d to produce

citrulline, which condenses with aspartic acid to form argininosuccinic acid, which is cleaved to arginine (77c).

Biosynthesis of tyrosine, phenylalanine, and tryptophan. The biosynthetic pathway to the three aromatic amino acids is summarized in 78a and given in detail in 79b, c, d, e. The six-membered ring that is the common precursor of the aromatic ring of each amino acid appears as shikimic acid (78b).

Its 5-phosphate derivative is then converted to chorismic acid which gives rise to prephenic acid, the common intermediate of tyrosine and phenylalanine, and to anthranilic acid, the precursor of tryptophan (78c). Tyrosine and phenylalanine are synthesized as shown in 78d, and the conversion of anthranilic acid to tryptophan is outlined in 78e.

Amino acid biosynthesis: Summary. Dia-

78.

(a)

Biosynthesis of Tyrosine, Phenylalanine, and Tryptophan

78. (cont.)

(c) СООН соон соон NH₂ anthranilic acid OPO₃H₂ COOH H₂C=C-COOH

phosphoenolI pyruvic acid H CH₂ CH_2 H₂O₃PO ∕OH H₂O₃PO -соон H H ÓΗ chorismic acid HO HOOC shikimic acid-5-P -СООН 3-enolpyruvylshikimic acid-5-P ÓН prephenic acid

78. (cont.)

(e)

gram 79 summarizes the biosynthetic pathways just discussed. It immediately becomes obvious that there are three principal pathways from the carbon skeleton of glucose (and other carbohydrates) to the carbon skeletons of the amino acids:

- (1) By way of the tricarboxylic acid cycle to α -ketoglutaric acid and oxalacetic or fumaric acids and thus to glutamic and aspartic acids.
- (2) By way of the pentose phosphate pathway to erythrose-4-phosphate.
- (3) By way of Embden-Meyerhof glycolysis to 3-phosphoglyceric acid and pyruvic acid.

The α -amino group is most commonly derived by:

- (1) Transformation of the carbon skeleton of glutamic or aspartic acids without loss of the α -amino groups.
- (2) Transamination of a precursor of the amino acid, most commonly its α -keto analogue.

With a few important exceptions, the

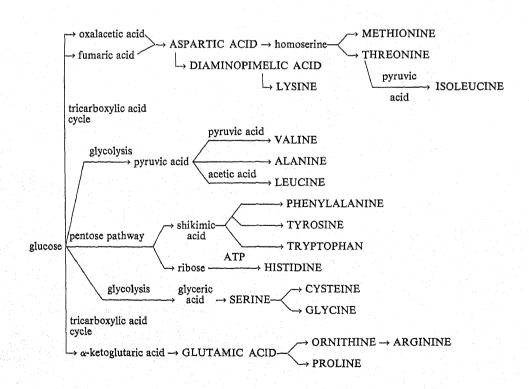
modes of amino acid biosynthesis appear to be the same in all bacteria.

Regulation of amino acid biosynthesis.^{1,8} In general, bacteria synthesize amino acids only when they have to. If a given amino acid is not supplied in the growth medium, a bacterial culture will synthesize it, but if it is added later, de novo synthesis stops at once. This prompt cessation of synthesis is brought about either by end-product inhibition, which is inhibition of the functioning of preexisting enzymes, or by enzyme repression, which is the inhibition of synthesis of new enzyme protein.

In end product inhibition, the final product of a reaction sequence (here an amino acid), characteristically inhibits the enzyme catalyzing the first step in the sequence unique to its synthesis, thus preventing the unnecessary functioning of the entire series of synthetic enzymes. For example, isoleucine strongly inhibits the first unique step in its synthesis, the formation of α -ketobutyric acid (72e), and histidine inhibits the first

79.

Biosynthetic Origin of the Amino Acids



step in its synthesis, the reaction between ATP and 5'-phosphoribosylpyrophosphate (76a). These amino acids act as negative effectors to combine with allosteric sites on the end product-sensitive enzymes to reduce their rates of enzymatic activity.

In repression of enzyme synthesis, the exogenously supplied amino acid acts as a corepressor to initiate a complex series of events that result in inhibition of the transcription of the information for the enzyme protein from DNA to messenger RNA (see Chap. Seven). In this manner, methionine may repress the synthesis of cystathionine synthetase (72d).

Synthesis of peptides. The basic chemical reaction in the synthesis of both peptides and proteins is the synthesis of the peptide

bond (see 80). The equilibrium of this reaction is far to the side of cleavage of the peptide bond, and therefore its synthesis requires an external source of energy. For all the peptides whose synthesis has been studied in detail, this energy is supplied by ATP, which reacts with the carboxyl group of one amino acid (or peptide) to form an amino acyl adenylate (see 81). This carboxyl activation of amino acids by ATP has more than a superficial resemblance to the carboxvl activation of fatty acids by coenzyme A. This activated intermediate then reacts with another amino acid (or another suitable acceptor) to form a new peptide bond (see 82 and the section on protein synthesis below.)

80.

81.

82.

Metabolism of Nucleic Acids, Nucleotides, and Related Substances

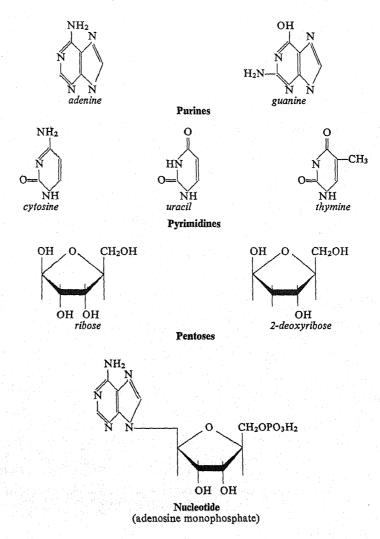
The main representatives of the second great group of naturally occurring nitrogenous substances are the nucleotides and the nucleic acids, which are high molecular weight substances made up of many nucleotide units. A nucleotide is composed of a purine or pyrimidine base, a pentose sugar which is either ribose or deoxyribose, and a molecule of phosphoric acid (see structural formulas in 83). Nucleotides are important

as metabolic intermediates and as portions of coenzymes such as ATP and ADP, NAD and NADP, and CoA. Nucleic acids are of two types. Deoxyribonucleic acid (DNA) contains the pentose deoxyribose and the nitrogen bases adenine, guanine, cytosine, and thymine. Ribonucleic acid (RNA) contains ribose instead of deoxyribose and uracil in place of thymine.

Bacteria and other microorganisms have

83.

Structural Components of Nucleic Acids



large amounts of nucleic acid as compared to higher forms. The nucleic acid content is highly variable and dependent upon the physiological state of the bacterial cell. Nucleic acids may occur in nature in combination with proteins as nucleoproteins. Nucleic acids and the various substances related to them contribute only a small portion of the total nitrogen of the usual bacteriological medium and are not generally a principal source of energy, carbon, or nitrogen. However, certain bacteria vigorously attack purines and pyrimidines and can use them as carbon and nitrogen sources. Also, some fastidious bacteria cannot synthesize all their purines and pyrimidines and must have them supplied in the growth medium.

BREAKDOWN OF NUCLEIC ACIDS³⁵

Nucleoproteins are easily dissociated into nucleic acid and protein, and no specific enzymes for this cleavage have been described. All cells, bacteria included, have numerous enzymes for degrading nucleic acids to smaller fragments. For example, *E. coli* has at least five enzymes that attack DNA. In both DNA and RNA, the nucleotides are linked in polynucleotide chains by 3'-5'-phosphate bridges (shown in 84). Nu-

84.

cleodepolymerases attack intact nucleic acids. Deoxyribonucleases (DNase) hydrolyze phosphate ester linkages in DNA. DNase I of pancreatic origin and streptococcal DNase (streptodornase) split 3'-bonds to yield 5'-mononucleotides and oligonucleotides with 5'-monophosphate ester end groups. On the other hand, DNase II, widely distributed in animal tissues and microorganisms, attacks 5'-bonds to yield 3 -fragments. All these enzymes fail to attack large portions of the DNA molecule, leaving a nonreactive core or limit polynucleotide.

Ribonucleases (RNase) are generally of a single type. They hydrolyze 5'-phosphate esters of pyrimidine nucleotides to yield mononucleotides representing almost all the pyrimidines present, di- and trinucleotides, and a limit polynucleotide.

Phosphodiesterases hydrolyze -O-P-O-bonds in RNA and DNA. RNA is attacked directly, DNA only after exposure to DNase. Phosphomonoesterases hydrolyze nucleotides to nucleosides and inorganic phosphate.

Nucleosides are cleaved to the free purine or pyrimidine base by rupture of the glycosidic bond between the nitrogen atom of the base and the sugar (N-glycosidic bond).

Like the glycosidic bond between two sugars, this N-glycosidic bond may also be split by phosphorylases or transglycosidases. These reactions will be discussed in connection with the synthesis of nucleosides and nucleotides.

Some clostridia, micrococci, and other soil organisms oxidize free purines and pyrimidines and may even be able to use them as sole sources of carbon. Purines are broken down to glycine, acetic acid, formic acid, and CO_2 , while pyrimidines yield β -alanine, lactic acid, acetic acid, and CO_2 .

The free *pyrimidines* are attacked by certain soil microorganisms and oxidized completely to CO₂ and H₂O. Barbituric acid, urea, and malonic acid may be intermediates in the oxidation.

The further metabolism of the *pentoses* released upon breakdown of nucleosides has been previously considered (see Carbohydrate Metabolism).

PRIMARY SYNTHESIS OF PURINES AND PYRIMIDINES

Although purines and pyrimidines are structurally related (see 83), they are synthesized by completely independent pathways.

Synthesis of purines.⁵ In 85 are shown the reactions in the biosynthesis of purines. This biosynthetic pathway leads to inosine

monophosphate, the ribose nucleotide of hypoxanthine. The free purine hypoxanthine is not an intermediate in the synthesis of its nucleotide because the purine ring is built onto ribose-5-phosphate, not vice versa. Following the initial condensation of glycine with 5-phosphoribosylamine, the remainder of the purine ring is formed by the stepwise addition of single atoms of carbon and nitrogen (see 85 and 86).

Synthesis of pyrimidines.³³ Diagram 87

85.

Synthesis of Purines

Origin of the Carbon and Nitrogen Atoms of the Purine Ring

CARBON
C4 and C5—glycine
C2 and C8—l-carbon
donors via tetrahydrofolic acid
C6—CO2

NITROGEN
N₃ and N₉—glutamine
N₁—aspartic acid
N₇—glycine

shows how pyrimidines are made. The synthetic mechanisms contrast sharply with those responsible for the production of purines. Aspartic acid and carbamyl phosphate condense to form the skeleton of the pyrimidine ring in one step, the ring is closed, and then the ribose-5-phosphate residue is added by reaction with 5-phospho- α -ribosylpyrophosphate. In an alternate pathway, β -alanine and carbamyl phosphate may condense to form uracil.

FURTHER METABOLISM OF PURINES AND PYRIMIDINES⁴⁰

Bacteria do not independently synthesize each purine and pyrimidine and its corresponding ribonucleosides and deoxyribonucleosides and nucleotides. Instead they carry out extensive interconversions among these different types of purine and pyrimidine derivatives.

A number of different kinds of enzymes are responsible for these interconversions. In the adenine series, as a specific example, adenosine monophosphate is formed from inosine monophosphate by a reaction (see 88) in which aspartic acid is the nitrogen donor (cf. synthesis of arginine from citrulline). It may be split to adenosine and inorganic phosphate by a specific nucleotidase. Adenosine in turn may be cleaved to free adenine and ribose by a nucleosidase. Adenosine monophosphate and adenosine diphosphate are formed by the interaction of ATP and adenosine.

adenosine + ATP
$$\frac{\text{nucleoside}}{\text{kinase}}$$
 AMP + ADP

Adenosine triphosphate is formed by oxidative phosphorylation as already described. The further metabolism of uridine monophosphate is shown in 89.

88.

inosine monophosphate

adenosine monophosphate

xanthosine monophosphate

guanosine monophosphate

89.

(a)
$$UMP + ATP \rightarrow UDP + ATP$$

 $2 UDP \rightarrow UTP + UMP$
(b) OH

uridine triphosphate

cytidine triphosphate

Transformations involving alterations in the ring substituents of the purine and pyrimidine bases occur in several ways. The amino group of adenine is deaminated to the hydroxyl group of hypoxanthine in free adenine, adenosine, or adenosine monophosphate. Guanine may be similarly deaminated to xanthine and hypoxanthine reversibly oxidized to xanthine. In the pyrimidines, cytosine is deaminated to uracil and thymidine is demethylated to deoxyuridine.

The principal pathway of deoxyribo-

nucleotide synthesis is the reduction of ribonucleotides to deoxyribonucleotides. In *E. coli*, ribonucleotide diphosphates are reduced to the corresponding deoxyribonucleotide diphosphates by a complex system involving two enzymes, a protein cofactor (thioredoxin), ATP, NADPH, and Mg⁺⁺. In other bacteria, there may also be a requirement for vitamin B₁₂ for the reduction of ribonucleotides to deoxyribonucleotides.

Thymine nucleotides, which exist only as the deoxyribonucleotides, are formed by

methylation of deoxyuridine monophosphate by a folic acid coenzyme and the enzyme thymidylate synthetase (see 90).

REGULATION OF THE BIOSYNTHESIS OF PURINES AND PYRIMIDINES

De novo biosynthesis of purines and pyrimidines in bacteria is strongly inhibited by the end products of these syntheses. Control of purine biosynthesis is achieved by regulation of the interconversion of the nucleotides of guanine, adenine, and inosine (see 88).²⁶ The conversion of inosine monophosphate to guanosine monophosphate is susceptible to end product inhibition by

guanosine monophosphate and repression by guanine, while the reduction of guanosine monophosphate to inosine monophosphate (which can then be aminated to adenosine monophosphate) is inhibited by ATP. Further control is exerted by the requirement of ATP for guanosine monophosphate synthesis and of guanosine triphosphate for synthesis of adenosine monophosphate (88).

Pyrimidine biosynthesis is controlled mainly by the inhibition of aspartyl transcarbamylase, which catalyzes the first step in pyrimidine synthesis, the formation of carbamyl aspartate (87). The most frequent end product inhibitor in bacteria is cytidine monophosphate, but other pyrimidine nucleotides may also be active.

Synthesis of Nucleic Acids and Proteins 9,41

All living cells contain the same low molecular weight building blocks produced by the same biosynthetic pathways. While some small molecules and some pathways occur uniquely in restricted groups of cells, variation in small molecule content is not an important determinant of the individuality of cells. Individuality, which is just as important a biological principle as similarity, is primarily a function of the macromolecules of the cells—DNA, RNA, and protein. They are polymers of common low molecular weight units, the nucleotides and the amino acids, but these units are assembled

in specific linear sequences, and it is in these unique sequential arrangements of a limited number of similar units that biological specificity resides.

The ultimate repositories of biological specificity (i.e., genetic information) are the nucleotide sequences of molecules of DNA, the self-replicating genetic material. In addition to making more molecules like itself, the DNA causes the formation of RNA molecules whose nucleotide sequences carry some of the information possessed by the DNA. These RNA molecules in turn bring about the synthesis of the protein

enzymes required for the energetic and biosynthetic functions of the cell. This section will be limited to a description of the biosynthesis of these macromolecules. Their genetic functioning is the subject of Chapter Seven.

Structure and self-duplication of DNA. According to the model of Watson and Crick, each DNA molecule consists of two polynucleotide strands coiled about a common axis. This double helix is held together by hydrogen bonds between pairs of bases. Because of structural limitations, hydrogen bonds are formed only between adenine and thymine and between guanine and cytosine (91), and ratios of adenine to thymine and of guanine to cytosine are always unity.

In a single strand of DNA, any sequence of nucleotides is possible, and hence a very large number of different sequences, each carrying unique genetic information, is also possible. However, if the order of bases in one strand is specified, the exact sequence in the other chain is simultaneously determined because of the restrictions on base pairing. Thus, each strand in a molecule of DNA is the complement of the other, and each molecule contains two strands but only a single code.

This complementary structure of the double helix is the key to the mechanism of duplication of DNA. According to the model of semiconservative replication, each of the two strands acts as a template for the formation of a new chain with complementary nucleotide sequence. The net result is that two new strands are produced, complementary to each other and each identical with one of the original strands as well.

Synthesis of DNA. Kornberg and his associates have shown that E. coli contains an enzyme, DNA polymerase, which catalyzes the formation of polynucleotides with properties very similar to DNA isolated from natural sources. The polymerization may be visualized as in 92. All four deoxyribonucleoside triphosphates are required. Pyrophosphate is released, and the energy of the nucleoside triphosphates is used to form the polynucleotide chain. Mg++ is necessary for the reaction, and a small amount of polymerized DNA must be present to act as a primer, presumably in the manner just postulated for DNA replication. The amount of new DNA synthesized is many times that added as primer.

The newly synthesized DNA has all the physical properties of natural DNA and is

91.

Pairing of Bases in DNA

92.

$$\begin{bmatrix} n_1 d \, TPPP \\ n_2 d \, GPPP \\ n_1 \, d \, APPP \\ n_2 \, dCPPP \end{bmatrix} \xrightarrow{\begin{subarray}{c} DNA \\ \hline template, \\ Mg^{++} \end{subarray}} \begin{bmatrix} d \, TP \\ d \, GP \\ d \, AP \\ d \, CP \end{bmatrix} + 2(n_1 + n_2)PP \\ d \, CP \end{bmatrix} \\ deoxyribo- \\ nucleoside$$

$$deoxyribonucleic\ acid$$

triphosphates

hydrolyzed by pancreatic DNase. The ratios of adenine to thymine and of guanine to cytosine are those required by the Watson-Crick model, and the base ratios of the new DNA molecules are very close to those of the primer. It has not been proved that the DNA polymerase of Kornberg is the enzyme that actually precisely duplicates DNA during bacterial reproduction, but it is highly likely that this duplication is carried out by enzymes with similar properties. The DNA of a bacterial virus has recently been made in a biologically active form in vitro through the action of DNA polymerase and one other enzyme.

Synthesis of RNA. Bacteria, plants, and higher animals possess an enzyme, RNA polymerase, which catalyzes the synthesis of RNA from all four ribonucleoside triphosphates, Mn⁺⁺, and a DNA primer (93),

The resemblance to the DNA-synthe-sizing system just described is striking. Here DNA also acts as a template for polynucleotide assembly. However, since the building materials at hand are ribonucleotides, RNA, not DNA, is produced. The template role of the primer DNA is clearly shown by observations that the composition of the newly synthesized RNA closely approximates that of the DNA primer, even when DNA molecules of widely varying base ratios are employed.

Although it appears that in vitro both

strands of the DNA helix may serve as templates for the synthesis of RNA, only one of the DNA strands is probably copied in the intact bacterial cell. RNA synthesized on a DNA template and carrying the genetic information of that portion of the DNA molecule to which it is complementary is called *messenger RNA* (mRNA). The process of transferring information from DNA to RNA is called *transcription*.

Ribopolynucleotides are also synthesized by the widely distributed polynucleotide phosphorylase. The enzyme polymerizes single ribonucleotide diphosphates or mixtures of them (94). Thus, polymers containing one, two, three, or four nitrogen bases may be synthesized. When the polynucleotide phosphorylase is purified, a primer becomes necessary. The requirement is for an oligonucleotide with a free 3'-hydroxyl group to which the new nucleotide units are added.

The genetic code.⁹ The information required for the insertion of a specific amino acid into a specific position in a polypeptide chain is contained in three successive nucleotide residues of a molecule of DNA and its complementary mRNA; that is, the genetic code consists of nucleotide triplets. The ultimate repository of information is in the triplets of DNA, but the complementary triplets, or codons, of mRNA are usually used to formulate the genetic code, which

93.

$$\begin{bmatrix} n_1 UPPP \\ n_2 GPPP \\ n_1 APPP \\ n_2 CPPP \end{bmatrix} \xrightarrow{\begin{subarray}{c} DNA \\ template, \\ Mg^{++} \end{subarray}} \begin{bmatrix} UP \\ GP \\ AP \\ CP \end{bmatrix} + 2(n_1 + n_2)PP \\ 2n_1 + 2n_2 \\ ribonucleoside \\ triphosphates \end{bmatrix}$$

94

$$\begin{bmatrix} n_{l}UPP \\ n_{2}GPP \\ n_{3}APP \\ n_{4}CPP \end{bmatrix} \xrightarrow{Mg^{++}} \begin{bmatrix} UP \\ GP \\ AP \\ CP \end{bmatrix} + (n_{l} + n_{2} + n_{3} + n_{4})P$$

$$= n_{l} + n_{2} + n_{3} + n_{4}$$

may be conveniently tabulated as shown in 95. This shows the best currently available allocation of the codons and is based mainly on results obtained with *E. coli*. Thus, the codon specifying phenylalanine is UUU, that specifying tyrosine is UAU or UAC, etc.

Synthesis of proteins. Protein synthesis requires a source of energy for formation of the peptide bond and a mechanism for ordering the sequence of amino acids in the polypeptide chain. As in the synthesis of DNA and RNA, the energy is supplied by a nucleoside triphosphate and the template by a polynucleotide. However, since proteins are made up of amino acids, not nucleotides, both processes are more indirect and more complicated.

Amino acids are activated in the form of aminoacyl adenylates by specific activating enzymes associated with the cytoplasmic membrane and soluble fraction of the bacterial cell. There is at least one activating enzyme for each amino acid. The first step is:

 $\frac{Mg^{++}}{amino\ acid + ATP + E} = \frac{(amino\ acyl-AMP-E) + PP}{amino\ acyl-AMP-E}$

	2nd								
1st	U	С	A	G	3rd				
U	phe	ser	tyr	cys	U				
	phe	ser	tyr	cys	C				
	leu	ser	ochre	?	A				
	leu	ser	amber	try	G				
c	leu	pro	his	arg	U				
	leu	pro	his	arg	C				
	leu	pro	glun	arg	A				
	leu	pro	glun	arg	G				
A	ileu	thr	aspn	ser	U				
	ileu	thr	aspn	ser	C				
	ileu	thr	lys	arg	A				
	met	thr	lys	arg	G				
G	val	ala	asp	gly	U				
	val	ala	asp	gly	C				
	val	ala	glu	gly	A				
	val	ala	glu	gly	G				

Key: U = uracil, C = cytosine, A = adenine, G = gua-

The next step is the transfer of the activated amino acid to a special kind of RNA, transfer RNA (tRNA).

amino acyl - tRNA + AMP + E

There is at least one tRNA for each amino acid. They are small polynucleotides, about 70 to 80 nucleotides in length, and the complete nucleotide sequence is known for a few of them. The tRNA molecules have two recognition sites, the one just described for recognizing the activating enzyme and another separate site, the coding site, for binding to a specific codon in the mRNA strand. All amino acids are linked to their transfer RNA's through esterification to the 2'- or 3'-hydroxyl groups of a terminal adenosine moiety.

Polypeptide chains are assembled on the surface of the *ribosomes* (see Chap. Three). These cytoplasmic particles contain about equal amounts of protein and a third species of RNA, *ribosomal RNA* (rRNA). In *E. coli*, the bacterium in which protein synthesis has been most extensively studied, the ribosomal particles involved in protein synthesis are 30 S and 50 S units. In the presence of mRNA, these units attach themselves to the polynucleotide strand to form polyribosomes or polysomes:

$$[30S + 50S]_x + mRNA \rightarrow polysome$$

The value of x is in the range of 5 to 20. The ribosomes of procaryotic cells must differ in subtle ways from those of eucaryotic cells, because protein synthesis on procaryotic ribosomes is much more susceptible to inhibition by antibiotics such as chloramphenical and the tetracyclines.

Although mRNA is the template on which the amino acids are assembled to be formed into polypeptide chains, it cannot function directly because polypeptides cannot be formed on polynucleotide templates. This difficulty is circumvented by the structure of tRNA. The coding site of the tRNA acts as an adapter; that is, each tRNA has a coding site complementary to a specific codon in the mRNA strand and binds to it, thus locating its activated amino acid in a definite relationship to other amino acids similarly bound. This transfer of information is known as translation.

During synthesis of a polypeptide chain,

ala = alanine, arg = arginine, asp = aspartic acid, aspn = asparagine, cys = cysteine, glu = glutamic acid, glun = glutamine, gly = glycine, his = histidine, ileu = isoleucine, leu = leucine, lys = lysine, met = methionine, phe = phenylalanine, pro = proline, ser = serine, thr = threonine, try = tryptophan, tyr = tyrosine, val = valine.

the mRNA moves across the ribosomes of its polysome unit to successively expose its codons, which bind in proper sequence the appropriate amino acyl-tRNA complexes. Polypeptide chains are always synthesized from their N-terminal ends.

The mechanisms of the movement of the mRNA across the ribosomes and of the formation of the peptide bonds of the polypeptide chain are not well understood. The energy for moving the mRNA strand and for bringing the amino acyl-tRNA into its proper binding position is probably supplied by the hydrolysis of guanosine triphosphate, which is required in all protein-synthesizing systems so far studied. The energy for peptide bond formation is provided by hydrolysis of the amino acyl complexes at the moment of peptide bond synthesis. This reaction is catalyzed by a specific enzyme (transfer factor).

There must be specific mechanisms for starting and stopping the synthesis of a polypeptide chain. In E. coli, most poly-

peptide chains, at the moment of their elaboration, are made with the common N-terminal sequence shown in 96, although all these residues may be removed in the final fashioning of the biologically active protein. It is highly likely that the codon that recognizes N-formyl-methionine-tRNA is responsible for chain initiation, but the structure of this codon is still in doubt. There are two codons that may, in certain systems, result in chain termination, apparently because they specify no amino acid at all. These are the amber (UAG) and ochre (UAA) triplets (see 95). These may be universally occurring natural chain terminators, but this is not certain.

The complicated interrelationships in the biosynthesis of DNA, RNA, and protein are shown in a greatly simplified and abbreviated diagram (97). Note that the ultimate source of genetic information is the sequence of bases in DNA and that the ultimate source of biosynthetic energy is the high energy bonds of ATP.

96.

97.

dNTP = deoxyribonucleoside triphosphate; NTP = ribonucleoside triphosphate.

Nutrition of Bacteria

In order to synthesize the many components of their protoplasm and thus to grow and multiply, bacteria must be supplied with a medium containing the proper chemical constituents for supporting the necessary biosynthetic reactions. The chemical substances which must be furnished bacteria in order that they may grow and multiply are known as their growth requirements or nutritional requirements. These nutritional factors are usually classified as:

- (1) Compounds required as energy sources.
- (2) Compounds required as building stones for the synthesis of new cell material
 - (a) Compounds required as carbon sources.
 - (b) Compounds required as nitrogen sources.
 - (c) Organic compounds required in their intact form as growth factors.
- (3) Inorganic ions required for metabolism and growth.

Given these necessary factors, bacteria will grow in the proper physicochemical environment (favorable temperature, pH, O₂ tension, etc.). Different kinds of bacteria have widely varying growth requirements, and even the same bacterium may have variable nutritional needs, depending upon the conditions of growth and the presence or absence of other substances in the medium.

Many aspects of bacterial nutrition have already been treated in terms of the metabolic functioning of the various nutritional factors. In particular, the sources of energy, carbon, and nitrogen have been repeatedly discussed and will not be considered further here. Much has also been said about the metabolic function of growth factors, so the chief object of this section will be to collect and summarize the basic information about the role of growth factors in bacterial nutrition

Growth factors. A growth factor may be defined as an organic compound which a bacterium needs for growth yet cannot synthesize. Since all cells have essentially the same chemical make-up, it follows that bacterium A, which does not need a growth factor required by bacterium B, lacks this requirement because it can synthesize the factor in question. Thus, L. casei requires folic acid, while E. coli does not because it makes its own folic acid.

It is generally believed that the variations in nutritional requirement observed among

presently existing groups of bacteria have arisen by loss of synthetic ability through genetic modifications. This idea receives direct experimental support from work in bacterial genetics (Chap. Seven) which has demonstrated that mutations in bacteria cause the loss of ability to synthesize those compounds—the B vitamins, amino acids, purines, and pyrimidines—which are growth factors for naturally occurring bacterial populations.

Growth factors are of two main types: (1) those required in minute amounts and which function catalytically as portions of enzyme systems: the B vitamins are the principal representatives of this type; (2) those required in substantially large quantities and which are incorporated directly or with only minor modifications into cell material: the amino acids, purines, and pyrimidines are the chief examples of this kind of growth factor.

An organism may exhibit an absolute requirement for a growth factor; i.e., no growth at all occurs in its absence. On the other hand, a growth factor may be stimulatory; i.e., limited growth without the factor is sharply increased upon its addition.

The vitamin B group.³⁴ The B-complex vitamins, or the vitamin B group, are low molecular weight, water-soluble organic compounds which are universal constituents of all living cells, in which they function as coenzymes or portions of coenzymes. It is customary to designate as the vitamin the simplest compound which, when supplied to the cell, allows it to synthesize the coenzyme. Thus, pantothenic acid is a B vitamin whose coenzyme form is CoA.

Since the B vitamins function catalytically, they promote bacterial growth in very low concentrations. The B vitamins are usually supplied to bacteria in concentrations of about 10^{-6} to 10^{-10} M or 0.0001 to 0.00000001 mg. per ml. of medium. Vitamin B₁₂ and biotin are generally required in smallest amount, nicotinic acid and riboflavin in the largest.

The accompanying table summarizes the metabolic function of the B vitamins.

Thiamin. Thiamin is one of the earliest recognized and most frequently required bacterial growth factors. It is synthesized by condensation of the thiazole portion

Metabolic Functions of the Vitamin B Group

B VITAMIN	TYPICAL ORGANISMS REQUIRING IT	COENZYME FORM	METABOLIC FUNCTIONS
Thiamin	Staph, aureus L. fermenti	DPT	Activation of keto acids and keto sugars; transfer of 2-carbon units
Nicotinic acid	L. arabinosus Pr. vulgaris	NAD and NADP	Hydrogen transfer
Riboflavin	L. casei Str. lactis	Riboflavin-5-P Flavin-adenine- dinucleotide	Hydrogen transfer
Vitamin B ₆			
pyridoxal pyridoxal or	L. casei {Cl. welchii	Pyridoxal-P Pyridoxamine-P	Decarboxylation, deamination, transamination, and racemization
pyridoxamine Pantothenic acid	\Str. fecalis Brucella abortus Pro. morganii	CoA 4'-Phosphopantetheine	of amino acids Acyl activation and transfer Fatty acid synthesis
p-Aminobenzoic acid	Cl. acetobutylicum Acetobacter suboxydans	Tetrahydrofolic acid	1-carbon-transfer
Folic acid	L. casei Cl. tetani	Tetrahydrofolic acid	r-carbon-transici
Biotin	Leuc. mesenteroides Cl. tetani L. arabinosus	Biotin-CO ₂	CO ₂ fixation, fatty acid synthesis
Vitamin B ₁₂	L. leichmannii L. lactis	$5'$ -deoxyadenosyl- B_{12} , methyl- B_{12}	1-carbon transfer Synthesis of deoxyribosides

and the pyrimidine portion of the molecule (see 98).

As expected from the way thiamin is synthesized, some bacteria require the thiazole alone, the pyrimidine alone, both thiazole and pyrimidine, or intact thiamin. The biosynthetic origin of the thiazole and pyrimidine intermediates is unknown. So far, no microorganism has been found with a requirement for the whole coenzyme, DPT.

Nicotinic acid (niacin). Nicotinic acid and nicotinamide are the growth factors

usually required by bacteria for the synthesis of NAD and NADP (see 4 for the

structural formulas). However, many species of the genus Hemophilus cannot utilize nicotinic acid or its amide for the

98.

synthesis of NAD and NADP and must be supplied with the intact coenzymes or with nicotinamide riboside. The probable metabolic pathway from nicotinic acid to NAD and NADP is shown in 99.

Riboflavin. Riboflavin as a growth factor resembles nicotinic acid, not in structure, but in the functioning of its coenzyme in hydrogen transfer and in the mode of synthesis of the coenzyme from the vitamin. Riboflavin has the structure shown in 100.

It is composed of a nitrogenous base, 6.7-dimethylisoalloxazine (flavin) and ribitol, the sugar alcohol corresponding to ribose, and is, therefore, comparable in structure to nicotinamide riboside. Its coenzyme forms are riboflavin-5-phosphate and flavin adenine dinucleotide (see 6 for structures).

The biosynthesis of the isoalloxazine ring is closely related to that of the purine ring. Note that the portion of the isoalloxazine ring lying to the right of the dotted line in 100 is identical with that of the purine ring (minus carbon 8). The riboflavin coenzymes are synthesized in a manner quite similar to that of the nicotinic acid coenzvmes (see 101).

Vitamin B_6 . Vitamin B_6 is a collective term for several closely related factors (structural formulas in 102). Of these, pyridoxal phosphate and pyridoxamine phosphate are the coenzyme forms (see Transamination). For higher animals, yeasts and molds, pyridoxine, pyridoxal, and pyridoxamine are of equal activity as growth factors. but for most bacteria, either pyridoxal or pyridoxamine is required. Some, such as

99.

(1) nicotinic acid + NH₃ → nicotinamide

(2) nicotinamide + ribose-1-P → nicotinamide riboside + H₃PO₄

(3) nicotinamide riboside + ATP → nicotinamide-5-P + ADP (4) nicotinamide-5-P + ATP → NAD + P-P

(5) NAD + ATP \rightarrow NADP + ADP

100.

101.

riboflavin + ATP → riboflavin-5-P + ADP riboflavin-5-P + ATP → flavin adenine dinucleotide + P-P

102.

The Vitamin B₆ Family CH₂OH CH₂NH₂ CHO НО HO / HO CH₂OH CH₂OH CH₂OH H_3C pyridoxine pyridoxal pyridoxamine CHO CH₂NH₂ HO CH2OPO3H2 HO CH2OPO3H2

pyridoxamine phosphate

pyridoxal phosphate

NUTRITION 171

L. casei, respond only to pyridoxal, while a few strains of lactobacilli require either pyridoxal phosphate or pyridoxamine phosphate.

Vitamin B_6 is a good example of a growth factor which is highly stimulatory, but not absolutely required. Most yeasts and lactobacilli such as L. arabinosus will produce heavy growth in the absence of vitamin B_6 , but in its presence the same amount of growth is achieved in a much shorter time.

Since it has been repeatedly demonstrated that pyridoxal phosphate is closely associated with almost every phase of amino acid metabolism, it is not surprising that the quantitative pyridoxine requirement depends upon the amino acid composition of the medium. This is particularly true for the lactobacilli which require many amino acids as growth factors. L. arabinosus can apparently synthesize a small amount of vitamin B₆, enough for growth on a medium rich in amino acids, such as a casein hydrolysate. However, omission of these amino acids forces the organism to make them itself, and it cannot synthesize enough vitamin B₆ to catalyze the reactions involved. Therefore, on a medium poor in amino acids, L. arabinosus must have additional vitamin B₆ for growth.

Pantothenic acid. Pantothenic acid is required for the growth of many bacteria. Its principal function is as a part of CoA, the coenzyme of acetyl transfer. It is synthesized by the union of β -alanine and pantoic acid, which are derived from aspartic acid and valine, respectively (see 103).

As with thiamin, some organisms require only one portion of the vitamin (β -alanine or pantoic acid) for growth, but the lactobacilli and many other bacteria can utilize only intact pantothenic acid.

One lactobacillus, L. bulgaricus, requires a higher form of pantothenic acid, pantetheine, formed from pantothenic acid and cysteine (see formula 104). It is very probable that pantetheine is an intermediate in the biosynthesis of CoA (see 35 and 105). 4'-Phosphopantetheine is the coenzyme of fatty acid synthetase. CoA is not an absolute growth requirement for any microorganism, but it is a stimulatory factor for Acetobacter suboxydans.

p-Aminobenzoic acid and the folic acid group. The role of folic acid in 1-carbon transfer has already been discussed in connection with the metabolism of amino acids and of purines and pyrimidines. Each member of the folic acid group contains p-aminobenzoic acid and a pteridine nucleus, and

103.

104.

pantothenic acid

β-mercaptoethanolamine

Pantetheine

they are metabolically interconverted by many bacteria. The precursor of *p*-aminobenzoic acid is 5-P-shikimic acid (see 78). Its amino group is donated by glutamine. The pteridine ring has a close structural resemblance to the purine and isoalloxazine rings, and all three are synthesized by related mechanisms.

p-Aminobenzoic acid is incorporated into folic acid, and the sulfonamides inhibit bacterial growth by preventing the incorporation.

Many bacteria, such as *E. coli*, do not require *p*-aminobenzoic acid or any of its higher forms, but its importance in their metabolism is easily demonstrated by its ability to overcome the growth inhibition produced by sulfonamides. Others, such as *L. arabinosus*, require preformed *p*-aminobenzoic acid but can synthesize the remainder of the folic acid coenzyme themselves. How-

ever, certain lactobacilli and clostridia also lack the ability to synthesize other portions of the folic acid molecule as well. Thus, Str. fecalis requires a growth factor of at least the complexity of pteroic acid, in which the bond between p-aminobenzoic acid and the pteridine nucleus has already been formed. L. casei lacks, in addition, the ability to join glutamic acid to pteroic acid and, therefore, requires pteroylglutamic acid as a growth factor. Finally, Pediococcus cerevisiae needs leucovorin, which differs from pteroylglutamic acid in the possession of a formyl group and a reduced pteridine nucleus. There are also many other forms of folic acid.

The coenzyme forms of folic acid are tetrahydrofolic acid and derivatives of this compound with active 1-carbon groups. Their probable biosynthetic origin is shown in the accompanying diagram (106). A gen-

106.

Biosynthesis of Folic Acid Coenzymes

NUTRITION 173

eralized formulation of the role of folic acid coenzymes in 1-carbon transfer is shown in 107. Other evidence also suggests that folic acid may be active in the synthesis of the amino acids histidine, threonine, and leucine.

Just as the vitamin B_6 requirement is profoundly influenced by the amino acid composition of the medium, so may the p-aminobenzoic acid or folic acid requirement be reduced or abolished by addition of the purines, pyrimidines, and amino acids which are synthesized in folic acid-requiring reactions. These same substances will also counteract the growth-inhibiting action of sulfonamides on organisms which require neither p-aminobenzoic acid nor folic acid.

Biotin. Biotin is a growth factor for many different types of microorganisms and for higher animals. It is likely that the pathway of biotin biosynthesis is as shown in 108.

In combination with a specific enzyme protein, biotin reacts with CO_2 to form an enzyme-biotin- CO_2 complex, which, as already discussed, acts as a CO_2 donor in several heterotrophic carbon dioxide fixations. The structure of biotin- CO_2 is shown in 109.

The biotin requirement of microorganisms, like that for the other B vitamins, is markedly affected by the presence of substances which are synthesized in biotin-dependent reactions. The best example of this dependency is furnished by the interrelationship of biotin, aspartic acid, and oleic acid in the nutrition of *L. arabinosus*. The bacteria will grow without either aspartic acid or oleic acid if the biotin concentration is high. If aspartic acid is also added, the biotin requirement is cut ten-fold; if both aspartic and oleic acids are added, *L. arabinosus* grows without any biotin at all in the medium.

Vitamin B_{12} .³⁹ This vitamin is a large and complex molecule consisting of (1) a complex ring system, the corrin moiety, closely related to the porphyrins of the cytochromes, chlorophyll, and hemoglobin and synthesized by similar mechanisms, (2) an atom of trivalent cobalt bound to the porphyrin-like structure in much the same fashion as the iron in hemoglobin, (3) a cyanide ion linked to the cobalt ion, (4) a nucleotide containing a substituted benzimidazole as the base, and (5) a molecule of an amino alcohol which is bonded to both the nucleo-

107.

(1) tetrahydrofolic acid + donor- $C_1 \rightleftharpoons$ tetrahydrofolic acid- $C_1 +$ donor

(2) tetrahydrofolic acid-C₁ + acceptor = tetrahydrofolic acid + acceptor-C₁

108.

109.

tide phosphorus and a side chain of the corrin moiety (see 110).

Although perhaps the greatest interest in vitamin B₁₂ stems from its ability to combat certain human anemias, it is required by many lactobacilli, and numerous enzymatic reactions in bacteria are dependent on coenzyme forms of vitamin B_{12} . For example, the previously discussed conversion of methylmalonyl-CoA to succinyl-CoA (49) and the reduction of ribonucleoside diphosphates to their deoxyribonucleoside counterparts require the participation of 5'-deoxyadenosyl-B₁₂ in which the CN⁻ of vitamin B₁₂ has been replaced with a 5'-deoxyadenosyl group derived from ATP. Another vitamin B₁₂ coenzyme, methyl-B₁₂, together with N5-methyltetrahydrofolic acid, is required for the methylation of homocysteine to methionine (72d).

Amino acids.²⁸ Many bacteria can synthesize all their amino acids themselves

(E. coli) or can synthesize all but one or two (Salmonella typhi requires only tryptophan). In general, the gram-negative organisms are potent amino acid synthesizers and require no preformed amino acids or only a limited number. The ability of gram-positive bacteria to synthesize amino acids is much more limited, particularly among the lactic acid bacteria. Leuc. mesenteroides requires, for example, 17 different amino acids.

Amino acids are required in substantially higher concentrations than are the B vitamins, roughly about 10⁻⁴ M or about 0.01 mg. per ml. of medium.

Amino acid assimilation. Since grampositive organisms lack the ability to synthesize many amino acids, they must take them up or assimilate them from the medium. Many gram-positive bacteria, perhaps in compensation for their lack of synthetic powers, have developed efficient mechan-

110.

cyanocobalamin

Vitamin B₁₂

NUTRITION 175

isms for assimilating and concentrating the amino acids of the medium within their cells. Thus, Staphylococcus aureus can assimilate glutamic acid until the intracellular amino acid level is 400 times that of the external medium. The exact mechanism by which this concentration is achieved is unknown, but it is an active, energy-requiring process. Not all amino acids are concentrated within the cell to the same degree as glutamic acid. Lysine, for example, enters the cell only by diffusion and thus never attains an intracellular concentration higher than that of the medium.

Factors influencing the requirement for amino acids. Although the amino acid requirement of an organism depends ultimately on its genetic composition, which in turn defines the range of its synthetic ability, the expression of this requirement varies under different conditions of growth.

- (1) THE EFFECT OF VITAMINS. The modification of the requirement for vitamin B₆, biotin, and folic acid by the presence of amino acids in the medium has already been discussed. It is obvious that the reverse also holds true. Thus, pyridoxal may alter the requirement for D-alanine, biotin for aspartic acid, folic acid for methionine, and so on.
- (2) THE EFFECT OF OTHER AMINO ACIDS. Many amino acids interfere with or antagonize the metabolism of other amino acids. For example, Bacillus anthracis grows without either leucine or valine. Yet if leucine is added, growth is inhibited, but is restored in the presence of both leucine and valine. These amino acids have similar structures, and it is probable that leucine acts as an inhibitor for the synthesis of valine, thus preventing growth until valine is also added. Similarly, tyrosine prevents the synthesis of the closely related phenylalanine by E. coli. These are examples of antagonisms between naturally occurring essential metabolites of the same nature as the antagonism between p-aminobenzoic acid and the sulfonamides. Many other examples of amino acid antagonisms have been reported.

The amino acid requirement for specific functions, such as the synthesis of an enzyme, may be different from those for growth. E. coli, for example, needs exogenous methionine, tyrosine, and asparagine to make arginine decarboxylase.

Purines and pyrimidines. Many bacteria,

especially the lactobacilli, are unable to synthesize the purine and pyrimidine bases which they need as constituents of their nucleic acids, nucleotides, etc. In general, the synthetic block appears to be in the formation of the purine or pyrimidine nucleus, and the majority of nitrogen-base requiring organisms are still capable of some interconversion of the bases. Thus, L. arabinosus can utilize either adenine. guanine, hypoxanthine, or xanthine as its sole purine source. Other organisms are more exacting and respond much more to one purine than to another. The classic example of a pyrimidine requirement is that of Staph. aureus for uracil.

Purine and pyrimidine requirements are modified by the same type of factors that affect the amino acid requirement. The interrelation between folic acid and thymine and the purines has already been mentioned, as has the interchangeability of vitamin B_{12} and thymidine and other deoxyribosides in the nutrition of the same bacteria. Antagonisms between the purine bases also affect the purine requirement. In particular, adenine and guanine each interfere with the metabolism of the other in a number of bacteria and yeasts.

The inorganic elements. The elementary constituents of the bacterial cell other than the carbon, hydrogen, oxygen, and nitrogen present in organic compounds must also be supplied in an adequate nutritional medium. It is customary to include in bacteriological mediums phosphate, sulfate, potassium, calcium, magnesium, manganous, and ferric ions. The need for these ions in metabolism is obvious. Organic phosphate compounds play a role in almost every phase of intermediary metabolism, and as phospholipids and nucleic acids are a part of the structural framework of the cell. Sulfate, or some form of sulfur, is needed for synthesis of the sulfur amino acids. Potassium, calcium, magnesium, and manganous ions are cofactors or activators in many enzyme sys-

Some organisms also have more specific inorganic requirements. Thus, the diphtheria bacillus gives optimum toxin production with an iron concentration of about 0.00014 mg. per ml. of medium. Higher or lower concentrations inhibit toxin production without affecting growth. Azotobacter specifically requires molybdenum for some phases of nitrogen fixation.

Undoubtedly other inorganic ions also appear in bacterial protoplasm and, therefore, must be present in the medium. However, it is very hard to show clearly that a particular ion is absolutely required for growth. This is because the inorganic ions are universal contaminants of all types of materials, so that it is almost impossible to free mediums of the last traces of an ion.

Microbiological assay.²⁰ An important practical application of the elucidation of the growth factor requirements of bacteria has been the development of methods for quantitative determination of vitamins and amino acids in terms of the growth response of microorganisms. The high degree of specificity and sensitivity attainable in microbiological assay have made this technique an invaluable tool in many fields of biological research.

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PHYSICAL AGENTS, BACTERICIDAL SUBSTANCES (DISINFECTANTS), AND CHEMOTHERAPEUTIC DRUGS

The separation of the environmental factors, that so profoundly affect the life processes of living cells, into two groups, one designated physical and the other chemical, is undoubtedly artificial in many instances. It is often difficult, if not impossible, to differentiate the physical from the chemical; the lethal effect of a bactericidal substance, for example, may be a result of a combination of both physical and chemical activity or its mode of action may lie in the borderland of surface and physical chemistry. Nevertheless, such a separation has a certain reality and is useful for purposes of exposition.

Bacteria, like all other living organisms, consist of protoplasm, a delicately balanced, heterogeneous mixture of various substances in colloidal and true solution. The disturbance of this equilibrium through, for instance, the precipitation of constituent protein is incompatible with the continuation of the complex phenomena of life, and the cell dies. On the other hand, the environment, when favorable, not only does not destroy this equilibrium but makes possible its continuation through growth and multiplication of the organisms. Bacteria, like other organisms, are creatures of their environment; under favorable conditions they multiply rapidly, and under unfavorable conditions either die or remain dormant in a viable state until another opportunity for growth presents itself. In general, they are much more resistant to unfavorable circumstances than are most higher forms of life. A part of this resistance is, of course, a result of the ability of some bacteria to form spores which are relatively highly resistant, but the vegetative cells are considerably more resistant than are the cells of multicellular organisms.

The inactivation of viruses tends to be set somewhat apart since they consist of nucleic acid protected, in the vegetative form, by a protein coat whose contribution to viral replication is not altogether clear, and they are not physiologically complete units like the bacteria. ^{26, 73} Thus bacteria could not survive the treatment with 50 per cent phenol, a good disinfectant, used to extract infectious ribonucleic acid from many RNA-containing viruses, nor are bacteria or vegetative viruses destroyed by treatment with ribonuclease as is infectious RNA.

It is not possible to differentiate sharply between these favorable and unfavorable factors. The congruity of a given environmental factor with the protoplasmic equilibrium that is life is largely a quantitative rather than a qualitative phenomenon. Although high temperatures destroy bacteria, a certain degree of warmth is essential to their growth. Distilled water is toxic to many microorganisms, yet multiplication takes place only in the presence of adequate amounts of moisture. Even highly active bactericidal chemicals often markedly stimulate growth of bacteria, when in sufficiently low concentrations.

Physical Agents

Temperature relations.80 Bacteria as a group will thrive under a relatively wide range of temperature conditions. Some of the more hardy, such as Bacillus subtilis, will grow throughout the range of 6° to 50° C. Others, such as many of the pathogenic forms, are able to grow over a much narrower range, and some of the more fragile organisms will grow only at body temperature, i.e., 37° C., or very close to this temperature. For all these organisms, however, three temperature limits may be distinguished. There is the minimum or lowest temperature at which a given organism will grow, an optimum or temperature of most luxuriant growth, and a maximum, the highest temperature at which growth can take place. The position of these three points differs greatly among different species of bacteria. In general, those organisms whose natural habitat is soil or water have optimum temperatures of 22° to 28° C., while those which, presumably as a result of adaptation to a parasitic mode of existence, cannot survive outside the animal body, have an optimum of 37° C.

There are, however, bacteria whose optimum temperatures differ considerably from these. Organisms have been found whose optimum temperatures are from 15° to 20° C., and they have been termed psychrophiles or cold-loving organisms. Others having optimum temperatures of 55° to 65° C. may be found in the soil and hot springs and are called thermophiles. The great majority of bacteria have optimum temperatures which lie between these two extremes and, in this terminology, are designated as mesophiles.

The optimum temperature for given species of bacteria is generally considered to be that temperature at which the organisms grow "best." The criteria of bacterial growth have been discussed elsewhere (Chap. Four). So far as the effects of incubation temperatures are concerned, those optimum for growth in terms of rapidity of cell division are not always the same as those optimum for the attainment of maximum numbers of cells per unit volume. A culture grown at a temperature at which cell multiplication is most rapid will not attain as high a peak in numbers as a culture of the same organism growing more

slowly at a lower temperature. 89, 93, 94 At higher temperatures oxygen is rapidly depleted to growth-limiting levels, and higher yields of cells grown at lower temperatures may be attributed to less rapid utilization coupled with greater solubility and availability of oxygen. 86

Other physiological activities of the cell appear to have optimum temperatures that differ from those which are optimum for multiplication. A given sugar may be fermented to a greater extent, more slowly, when the culture is incubated at a temperature somewhat below that optimum for cell division. Similarly, the anthrax bacillus forms spores most abundantly at 30° to 32° C., while its optimum for vegetative multiplication is 37° C.

The continued growth of bacteria at temperatures somewhat higher than optimum may induce physiological changes of a temporary or permanent character. Serratia marcescens, for example, fails to form its characteristic red pigment when incubated at temperatures higher than 30° C., but the change is temporary, for subcultures incubated at lower temperatures form pigment normally regardless of how many transfers have been grown at the higher temperature. The anthrax bacillus, on the other hand, when grown for several transfers at 42° C., loses its ability to form spores and becomes avirulent—a change that appears to be permanent. Incubation or storage of bacterial cultures at temperatures lower than optimum does not result in such qualitative physiological changes; the metabolic activities of the organisms are slowed down and at temperatures below the minimum for growth the bacteria become dormant.

The mechanisms determining the optimum, minimum, and maximum temperatures of bacteria are obscure. In some cases they may be dependent upon other environmental factors. Thus, while many of the thermophilic bacteria are able to grow only at temperatures above 50° C. when in contact with air, they are able under anaerobic conditions to grow at the ordinary incubator temperature (37° C.) or even as low as 34° C. There is evidence too that the maximum growth temperatures of bacteria bear a definite relationship to the minimum temperature of destruction of respiratory enzymes.

179

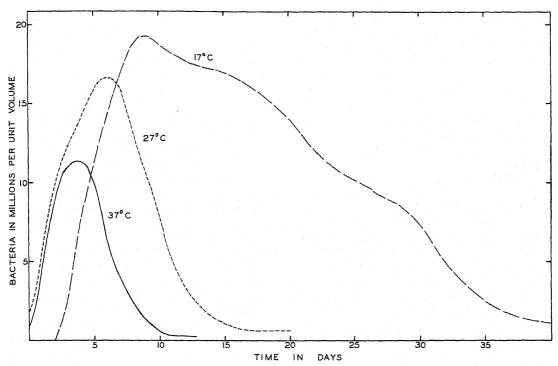


Figure 46. The effect of different temperatures of incubation on broth cultures of *Staphylococcus aureus*. (After Graham-Smith.)

The enzymes of thermophilic bacteria do not, however, have unusually high inactivation temperatures; rather, thermophilic growth is possible because the rate of synthesis of enzymes and other thermolabile cell constituents exceeds the rate of their destruction at the high environmental temperatures.⁴ The upper temperature limit of growth has been found to be 73° C.⁵⁶

The lethal effects of heat. Bacteria are readily killed by heat, and the utilization of heat in one form or another is one of the most convenient means of their destruction. The lethal effects of heat are markedly influenced by the amount of moisture present. and so-called moist heat is a much more effective killing agent than dry heat. The resistance of bacteria to moist heat differs somewhat from species to species, the pathogenic forms being in general somewhat less resistant. The resistance of a given species is influenced by two important factors: the ability of the organisms to form spores and the previous history of the culture. Spores are always much more resistant than vegetative forms. Some species when in the spore stage can withstand the temperature of boiling water for upward of 16 hours. The vegetative forms of most bacteria, on the other

hand, are killed at 55° to 58° C. by 30 minutes' exposure in the presence of moisture.

The time and temperature of incubation influence, to some extent, the heat resistance of vegetative cells. Actively growing cultures in the logarithmic phase are generally somewhat less resistant than are cells removed from cultures containing maximum numbers of viable organisms. The temperature of incubation appears to affect thermal resistance somewhat, in that cultures grown at and above the optimum temperature are more resistant than cultures grown at suboptimal temperatures.

The pH of the liquid in which the microorganisms are suspended is of considerable quantitative importance. Deviations from neutrality increase, to a marked degree, the rate at which a given temperature kills bacteria. Acid foods, such as tomatoes, are more readily preserved than foods which have a neutral reaction. Likewise, the addition of sodium carbonate to water in which surgical instruments are boiled increases the efficiency of the heat and at the same time tends to reduce rusting.

The death of an organism from heat is determined not only by the temperature reached but also by the time of exposure.

The tubercle bacillus, for example, is killed by 30 minutes' exposure at 58° C., 20 minutes' at 59° C., and 2 minutes' at 65° C. One may, therefore, determine the thermal death point of a given bacterium by exposure to varied degrees of heat for a constant time, or, similarly, a thermal death time may be determined by holding the temperature constant and varying the time of exposure. Both are useful, the latter possibly somewhat more so.

The effect of heat seems to be injurious even when bacteria are not killed, since the cells that have been heated appear to require a longer period of germination. Exposure to sublethal heat prolongs the latent period preceding cell division without affecting the rate of regeneration of the respiratory function, with no evidence of a period of "recovery" distinct from growth. On the other hand, sublethal heating of bacterial spores often has the effect of accelerating germination.

Sterilization by heat. The application of moist heat to the destruction of bacteria may take several forms. Sterilization by means of steam under pressure is the most efficient of these, because it makes possible temperatures higher than 100° C. in the presence of moisture. At 15 pounds pressure, for example, the temperature will be 121.3° C., and at 20 pounds, 126.2° C. As a rule, exposure to 120° C. for 15 minutes suffices for the complete destruction of both vegetative and spore forms of bacteria, although rarely highly resistant spores may be found that are not killed by this exposure. Boiling, i.e., exposure to 100° C., suffices to kill all vegetative forms of bacteria within a few minutes, but sterilization is not effected, for the spores of many of the saprophytic bacilli are not destroyed by boiling for many hours. Since very few of the pathogenic bacteria form spores, boiling contaminated water for a few minutes renders it safe for drinking purposes, and the boiling of surgical instruments generally suffices to kill the vegetative cells of pathogenic bacteria, but not the spores of spore-forming pathogens such as those of the tetanus and gaseous gangrene bacilli.

Temperatures as high as 100° C. are not necessary for the destruction of vegetative forms of bacteria, since most of them are killed at 55° to 58° C. if exposed for 10 to 30 minutes. A vacuum-steam method of

disinfection, especially applicable to textile products such as blankets and clothing, has been described² in which the autoclave is evacuated to 20 mm. Hg and steam run in to the desired temperature. A temperature of 70° C. for 12 minutes killed vegetative cells, but a higher temperature, 105° C., was required to kill anthrax spores. In the preparation of vaccines, suspensions of bacteria are usually heated to 60° C. for 30 minutes to one hour (to allow an adequate margin of safety). The tubercle bacillus, Brucella abortus, and other pathogenic organisms occurring in milk are killed by the process of pasteurization, i.e., heating to 142° to 145° F. for 30 minutes.

Dry heat is much less effective as a germicide than moist heat. Temperatures of 160° to 170° C. must be maintained for two to three hours in order to ensure complete destruction.

Sterilization by heating is a common and useful procedure, and the nature of the material to be sterilized determines whether moist heat or dry heat is used. Bacteriological mediums, solutions, etc., are sterilized by steam under pressure in an autoclave, provided that the contained substances are heat-stable, while laboratory glassware, syringes, hypodermic needles, etc., are usually sterilized by dry heat in a thermostatically controlled oven (see Chap. Two). The temperature is ordinarily kept constant, 120° C. and 170° C. respectively, but the time required for sterilization depends upon the volume of liquid, how closely the containers are packed, and similar factors that affect penetration of heat to effective sterilizing temperatures.

The destructive effect of high temperatures on bacteria is apparently associated with the coagulation of constituent protein. The rate of heat coagulation of protein in solution closely parallels the rate of destruction of bacteria by hot water. Furthermore, the effect of the water content of egg albumin on the temperatures necessary for coagulation is similar to the observed relative efficiency of moist and dry heat in the killing of bacteria.*

^{*}Egg albumin in aqueous solution is coagulated at 56° C.; with 25 per cent water content at 74° to 80° C.; with 18 per cent water content at 80° to 90° C.; with 6 per cent water content at 145° C. Anhydrous egg albumin may be heated to 170° C. without coagulation,

COLD 181

Cold. In general, cold has a preservative effect on microorganisms in that, as the temperature is lowered from the optima for growth processes and other physiological activities, the metabolic rate is reduced. The temperature coefficient is substantially the same as that of other living organisms, i.e., the metabolic rate is reduced by approximately half for each 10° C. drop in temperature over the relatively narrow range in which the effect is demonstrable. Thus, microorganisms grow and metabolize rapidly at incubator temperatures, but tend to persist in a semidormant state at usual refrigerator temperatures (2° to 8° C.), and storage in the refrigerator is a common method of preserving bacterial cultures over a limited time.

The effect of chilling is not invariably preservative. Some microorganisms, such as the influenza virus, are still inactivated sufficiently rapidly at refrigerator temperatures that they may be preserved for only a few hours. Still other microorganisms, notably the gonococcus and meningococcus, tend to die out even more rapidly at refrigerator than at incubator temperatures.

Extreme low temperatures. It is commonly said that microorganisms survive freezing and extremely low temperatures such as those of liquid air (-190° C.) and liquid hydrogen (-250° C.). The effect of exposure to, and storage at, temperatures below the freezing point of water is undoubtedly injurious to a greater or lesser extent, but when some, even relatively few, of the millions of individual cells present are found to be viable, the microorganism is said to "survive" the treatment. Many, perhaps very many, are killed as a consequence of the operation of lethal factors, and the significant variables are the rate of freezing, the temperature at which the frozen cells are stored, and the rate at which they are thawed.64

When the cells are frozen slowly there is a preferential formation of extracellular crystal nuclei, and their growth produces an increase in extracellular osmotic pressure, withdrawing water from the cell which is added to the growing crystals. The freezing point of the remaining water is depressed by the increasing concentration of soluble substances as the temperature is reduced; the unfrozen water decreases from as much as 50 per cent of the total at the freezing point to less than 10 per cent at -10° C.

When the freezing is extremely rapid, the tendency to extracellular crystallization diminishes and intracellular crystallization occurs. In the first instance two lethal effects are operative, the one that of high electrolyte concentration and the other dehydratior denaturation. Very rapid freezing minimizes the first effect, but not the second.

The lethal effects are time and temperature dependent, declining exponentially with reduction in temperature and extrapolating to zero at the glassy transformation temperature (-130° C). There is a third potentially lethal factor affecting material that has been frozen sufficiently rapidly to give intracellular ice crystals; that of growth of the intracellular crystals, which is also directly and exponentially related to temperature. The lethal effects of these factors are also species-dependent in that some kinds of microorganisms are less affected by them than others.

In practice, storage at a temperature of -50° C. or less is required for prolonged viability. For example, the cholera vibrio, one of the more delicate bacteria in this respect, dies off within a few hours when stored as a frozen suspension at -25° to -30° C., but remains viable for at least two years at -65° C. So the ordinary "deep freeze" is not cold enough for storage of frozen bacterial cultures, but a dry-ice refrigerator, or its equivalent, is.

The sequence of events occurring in the course of freezing is reversed in the thawing process, with the rates of denaturation and crystal growth increasing exponentially as the temperature rises. The process is, in fact, more rigorous than that of freezing because of the kinetics of heat exchange, and rapid thawing is required to minimize lethal effects. It is obvious that the effects of these three phases of the process—freezing, storage, and thawing—cannot be separated from one another but may be evaluated only relatively when two are held constant and the third varied.

Drying. The vegetative forms of most bacteria are killed by drying in air, although the different species exhibit pronounced differences in their resistance. The tubercle bacillus is one of the more resistant and the cholera vibrio one of the more sensitive to drying. In general, the capsulated organisms are more resistant than the noncapsulated forms. Spores are quite resistant to drying;

the spores of the anthrax bacillus, for example, will germinate after remaining in a dry condition for 10 years or more. The resistance of the pathogenic forms causing disease of the upper respiratory tract is of particular interest in connection with airborne infection, for the length of time a droplet remains infective is a function, primarily, of the resistance of the particular microorganism to drying.29 The observed lethal effect of suspension of microorganisms in air is largely a function of relative humidity, and it has been found that maximal death occurs at about 50 per cent relative humidity. This is apparently a consequence of the amount of water retained in the droplets; at very low humidities the rapid desiccation renders the organism resistant to the toxic action of soluble substances concentrated by the partial drying, while at high humidities sufficient moisture is retained to keep such substances from reaching toxic concentrations.

Freeze-drying. 35, 101 The resistance of a given organism to drying is apparently determined to some extent by the rapidity of the drying process and the temperature at which the organisms are dried and stored. If bacteria are frozen rapidly, as with dry ice and alcohol or one of the glycols, dried from the frozen state (the lyophile process). and the container or ampoule evacuated and sealed, they remain viable with antigenicity and virulence unimpaired for months and years when stored in the refrigerator. It has been calculated, by extrapolation, that the survival time ranges from decades for vibrios and Acetobacter to centuries for staphylococci under these conditions.32 Most of the bacteria are killed in the process, with less than 1 per cent surviving,25 and it is these few survivors that grow on subculture. Probably the most important single factor in survival is the menstruum in which the bacteria are suspended. Poor results are usually obtained with ordinary broths, and the addition of normal serum, lactose, and other ingredients contributes to survival.31 Survival has been of particular interest in the preparation of BCG vaccine in a stable dry form; the inclusion of sodium glutamate apparently contributes considerably to the survival of this microorganism (Chap. Thirty-one).36, 81

Radiation.^{58, 59, 72, 102} There is little biological effect of visible light on non-chloro-

phyll-containing microorganisms except in the presence of certain dyes which increase absorption of light, and energy, to produce inactivation. This appears to be due in large part to the formation of peroxides and is inhibited in the presence of catalase. The phenomenon is called photodynamic sensitization.

Beginning in the ultraviolet region, the biological effects of radiation become pronounced, and the well-known bactericidal activity of direct sunlight is due to its content of ultraviolet light. The effects of ionizing radiation, x-rays and γ -rays (short x-rays) being the most commonly used, are even more marked.

With relatively large doses of radiation the microorganism is inactivated in that it will no longer reproduce, or, in the case of pathogens, produce infection, although overt evidences of viability such as respiration rate are not affected appreciably. With smaller doses, not sufficient to inactivate, the genetic mechanisms of irradiated microorganisms become unstable, and the mutation rate is increased from the normal spontaneous rate of perhaps 10⁻⁸ to as much as 10⁻³. Similar doses applied to lysogenic bacteria activate the latent infection with provirus so that complete virus is produced and the host cells are lysed as described elsewhere (Chap. Four).

Both ultraviolet and ionizing radiations are electromagnetic waves emitted in quanta, but they differ in the way in which biological effects are produced because they are absorbed differently, and because their quanta carry greatly different amounts of energy.

Ultraviolet radiation. Absorption in the region between 2500 and 3000 Å is due in part to the aromatic amino acids and cystine, with the peak at 2800 Å attributable to tryptophan and tyrosine. The peak absorption by nucleic acids, i.e., the purine and pyrimidine rings, occurs at around 2600 Å. Nucleic acids absorb much more strongly than proteins, and 25 to 50 times more energy is absorbed per unit weight by the former than by the latter. It is more than coincidence that the region of maximal absorption in the ultraviolet is also that of maximal antimicrobial activity.

At 2537 Å, the most effective and commonly used wave length, a quantum of ultraviolet radiation has an energy of about 5 ev. This is not sufficient to eject an elec-

RADIATION 183

tron to form an ion, a process which requires an average of 30 ev in biological material. but is enough to raise a constituent electron to a higher state of energy and thus produce excitation rather than ionization. Modification of molecules occurs by the breaking of bonds (for example, a covalent bond may be broken by about 4 ev), but since such events occur at random in the sense that they do not necessarily affect essential functional mechanisms, the effects of ultraviolet radiation are less than those of ionizing radiation. The surrounding medium is affected also, and the formation of peroxides contributes to the antimicrobial effects of irradiation. In total, about 100 times as much energy is required to inactivate microorganisms by ultraviolet radiation as by xradiation, and it is calculated that about 4 million quanta are necessary to inactivate a colon bacillus.

The effects of ultraviolet radiation are not completely established immediately, and for some time, even as much as several hours, they may be modified by exposure of the microorganisms to visible light. This phenomenon is that of photoreactivation.¹⁸ The mechanisms involved are far from clear, and two kinds of hypotheses are current. The exposure to visible light may, in some obscure way, repair damage caused by the inactivating radiation, or it may provide a possible alternative mechanism to replace that which was damaged. Reactivation may also occur following short exposure to heat (45° C.) or the addition of substances such as catalase, peroxidase, or some organic substances.

Ionizing radiation. Ionizing radiation includes α -rays (nuclei of helium atoms), β -rays (fast-moving electrons), γ -rays, and x-rays. As ionizing radiation passes through a microorganism, it collides with successive atoms to eject electrons and so create columnar zones of intense ionization. Secondary ionization, produced by ejected electrons along different paths (δ -radiation) may be of quantitative significance, especially with α -radiation.

The resistance of microorganisms, both bacteria and viruses, is relatively high, 10⁶ rads* or more being required for 99 per cent

inactivation. There is reason to believe that radiation effects may be a consequence of a direct hit of key substances within the cell, and their destruction or alteration by the intense ionization. This is the target theory, but it is not fully consistent with radiation protection.⁴⁶

The effects of radiation are modified in various ways. The oxygen effect, *i.e.*, the reduced radiosensitivity of a microorganism when oxygen is displaced with an inert gas or removed by metabolism, is well known. Similarly, certain compounds, especially those containing sulfhydryl groups, give excellent protection against radiation damage.

Radiation damage. The effects produced are basically biochemical in nature and appear to revolve to a considerable extent about injury to the genetic mechanisms, including the blocking of DNA synthesis. Other synthetic reactions, notably the formation of adaptive enzymes (Chap. Seven), are inhibited also. The processes of cell division appear to be affected more readily than at least some synthetic processes, and inactivated rod-shaped bacteria, for example, continue to grow for a time in filamentous form but do not reproduce. The marked increase in mutation rate resulting from irradiation in doses less than those required for inactivation has been taken to suggest that inactivating doses may give rise to lethal mutations which prevent subsequent multiplication of the irradiated cells.

Radiation-pasteurization. 7, 68 Inactivation of microorganisms by irradiation has some practical application in the preparation of vaccines and possibly also in the preservation of food. There is some reason to believe that vaccines prepared by radiation-inactivation of pathogenic microorganisms, such as dysentery bacilli and poliovirus, are superior immunizing agents because this kind of inactivation produces less change in significant antigenic substances than treatment with heat or disinfectants such as formaldehyde. Ultraviolet radiation has been used for this purpose but, because its lack of penetration poses some technical problems in the application of uniformly inactivating but not excessive doses, has not been widely used.

The application of irradiation to the preservation of foods has been of considerable interest. Radiation having high penetration is required, and of the several types, x-ray

^{*}A rad is defined as the amount of radiation required for the absorption of 100 ergs of energy per gram of substance having unit density.

and y-ray have practical applicability in this respect. X-rays may be generated in the conventional ways, and Co⁶⁰ can be used as a source of γ -radiation. The spores of Clostridium botulinum, routinely used for evaluating many food preservation procedures, are relatively resistant to radiation, and it has been found, for example, that 106 to 10⁷ rads is required for the destruction of the spores in meat to the acceptable standard of efficacy.55 While there is no residual radioactivity of any consequence, undesirable flavors are often produced which can be only partially eliminated by devices such as the incorporation of acceptable free radical acceptors. At lower dosages partial destruction is achieved which may have some utility in supplementing other procedures such as refrigeration.

Electrophoresis. Microorganisms are apparently unaffected by the passage of electric currents through their suspensions, provided that the effects of heating and acidity and alkalinity in the vicinity of the electrodes are controlled. They do, however, carry a charge that is a composite of those of the amphoteric substances comprising them, and at neutrality they are negatively charged, with an iso-electric point at pH 3 to 4. At alkalinities above the iso-electric point they move in the electric field toward the cathode, and this is known in the older literature as cataphoresis rather than electrophoresis. Bacteria are too large to move freely in a supporting medium such as paper, but certain of the viruses are separable by paper electrophoresis.

Cell disintegration. The bacterial cell may be broken up by physical means in a number of ways. The cells may, for example, be dried, commonly with acetone, or frozen,

and ground with alumina. The cell structure may also be broken down by the grinding action developed in shaking vigorously with small glass beads or similar particles. This is the principle of the Mickle disintegrator which shakes a mixture of bacteria suspension and small glass beads rapidly at the ends of a tuning fork. Or bacteria may be broken up by subjecting them to sudden changes in pressures of the order of 500 to 600 atmospheres, by sudden application or release of the pressure,24 but they are killed without disruption by the application of very high pressures, such as 10,000 atmospheres, which also denature proteins. Methods such as these split the cell wall, allowing the constituents of the cell to escape, and are used for the preparation of cell walls as well as for the study of intracellular enzyme activity, etc.

Still another method of breaking up the cell is treatment with sonic or ultrasonic vibrations. Sonic vibration at 9 to 10 kc. breaks up fragile bacteria, such as the cholera vibrio, in a few minutes, but others, such as lactobacilli, require longer treatment. Ultrasonic vibration at 30 to 100 kc. with reasonably large power input, perhaps 400 w., breaks up the cells much more rapidly. In either case there is a marked tendency to fragment the cell wall also, and some degree of denaturation of enzymatic activity often occurs.

Such physical destruction of the bacterial cell is, of course, lethal in that the fragmented cells are killed, and viable cells are rapidly reduced in number, but complete sterilization is extremely difficult to attain by such methods. There seems to be as yet no generally accepted theoretical basis for the effects of ultrasound.³⁹

Disinfectants 71, 78, 99, 102

Many kinds of substances have a deleterious effect on microorganisms. Antimicrobial substances have two kinds of activity, the one bactericidal, germicidal, or virucidal, and concerned with the killing of microorganisms, and the other bacteriostatic or growth-inhibiting. The line of demarcation between the two kinds of effects is not a sharp one, in that a bactericidal substance may inhibit growth rather than kill in low concentrations or when the period

of exposure is limited. It is quite generally observed also that concentrations just below those at which an antimicrobial effect becomes apparent markedly stimulate bacterial growth.

In a general way, a substance is regarded as primarily bacteriostatic when the range of concentration over which inhibition of growth occurs is a relatively wide one, and primarily bactericidal when it is narrow. Similarly, when the rate of killing is slow,

the exposed microorganisms may survive, in diminishing numbers for a considerable time without growth, and, for these survivors, the antimicrobial activity is growth-inhibiting. Conversely, when the rate of killing is rapid, and survivors are detectable for only a short time, the activity is regarded as primarily lethal. The distinction between lethal and growth-inhibiting activity is, therefore, two-fold in that it is dependent upon both concentration and rate effects and upon their interrelationship. So the distinction is one of degree rather than kind and is clear only at extremes.

The terms antiseptic and disinfectant are applied to describe antimicrobial activity. Etymologically, both imply the effective removal of pathogenic microorganisms, but by usage they have come to have somewhat different meanings. An antiseptic is a substance that has sufficient antimicrobial activity to interfere with the development of infection, but which is nontoxic when applied superficially to living tissue. A disinfectant is a more potent substance of marked antimicrobial activity which destroys all, or nearly all, pathogenic microorganisms but, because of associated toxicity, is applicable only to inanimate material. In each case the terms must be defined with respect to the microorganism against which the substance is tested or expected to be used, for a given substance may be an effective disinfectant for one kind of microorganism but not another. The term disinfection is also broadened to cover the process of destruction of pathogenic bacteria by either chemical or physical means.

Antimicrobial activity is a property of a wide variety of substances, both inorganic and organic in nature, and the exploitation of combinations of antimicrobial activity and toxicity and/or corrosive properties is a matter of considerable practical importance. Those having sufficiently low toxicity to allow them to be injected parenterally into the diseased host to reach and maintain effective antimicrobial concentrations in the tissues are the chemotherapeutic drugs, and are discussed in the following section; those that are useful as disinfectants and antiseptics are considered here.

Inorganic compounds. A number of inorganic substances have antimicrobial activity because of the toxicity to microorganisms of ions into which they disso-

ciate, or because of their activity as oxidizing agents to bring about some degree of cold combustion of cell substance.

Acids and alkalis. Both strong acids and strong alkalis, i.e., those that are highly dissociated, exert a marked bactericidal effect. The lethal activity of the mineral acids is associated with, and proportional to, the degree of their dissociation, but that of the organic acids appears to be an effect of the whole molecule, for the degree of dissociation is, as a rule, not great. The disinfectant action of alkalis such as sodium hydroxide is likewise proportional to the degree of dissociation. The germicidal activity of the hydroxides of the alkaline earths is, however, greater than can be accounted for on the basis of dissociation, for the metallic ion is often toxic in itself. Both acids and alkalis, in too low a concentration to kill bacteria rapidly, often enhance the activity of other disinfecting agents. For example, the germicidal activity of many salts is greater in the presence of acid or alkali, and, as noted above, bacteria are killed much more rapidly by heat in the presence of dilute acid or alkali than at neu-

The relation of bacterial growth to the acidity or alkalinity of culture mediums has been discussed elsewhere. Concentrations of hydrogen or hydroxyl ions compatible with growth are very low, of the order of 10⁻⁴ to 10⁻⁹ mols of hydrogen ions per liter. Almost all bacteria will grow at pH 7.0 $(1 \times 10^{-7} \text{ mols hydrogen ions per liter})$ but grow best at an optimum which varies from species to species. The minimum and maximum limits between which growth takes place likewise vary widely with species. Certain organisms such as the lactobacilli and Streptococcus lactis are termed aciduric organisms because they are able to grow profusely at pH 4.0 or less. Perhaps the most acid-resistant organism known is Thiobacillus thiooxidans. This organism accumulates sulfate in its cultures as an end product of the oxidation of sulfur and continues to grow even in the presence of N/10 sulfuric acid (Chap. Five).

Salts. Salts have, in general, two effects on bacteria. In very low concentrations they markedly stimulate growth and at higher concentrations they become toxic. The particular concentrations at which these effects are apparent are dependent upon the degree of dissociation of the salt, the

nature of the anion and the valency and molecular weight of the metallic ion. In general, the bivalent cations are more toxic than the monovalent cations, and the salts of the heavier metals are more toxic than those of the lighter metals. There is, however, no precise quantitative relation in either case.

The most active of the heavy metals are mercury, silver, and copper. Mercuric chloride is highly active in 0.1 per cent aqueous solution but the activity is bacteriostatic in a sense in that the treated cells may be "revived" by removal of the mercuric ion with hydrogen sulfide. In general, relatively few mercury compounds have antibacterial activity, and those which do as a result of the presence of mercuric ion are primarily bacteriostatic rather than bactericidal. The silver salts, such as silver nitrate, although somewhat less active, are still highly efficient germicides. Copper salts are still less active but are highly efficient in the destruction of algae and other chlorophyll-containing organisms. Dissociation of these salts is intimately related to their disinfectant properties. A solution of mercuric chloride in absolute alcohol has substantially no antibacterial activity, but if water is added the activity of the solution increases in proportion to the amount of water added. Because of the importance of ionization, a comparison of the bactericidal power of the various metallic salts on the basis of percentage solution is misleading; equimolecular solutions must be used and the ionization constants taken into consideration. The antimicrobial activity of the heavy metal salts is a result of the affinity of the cations for protein material; when the constituent protein of a bacterial cell is precipitated as an insoluble proteinate, the cell dies. Other factors appear to be involved also, however. It has been observed that inorganic metallic salts which are only slightly bactericidal individually may become markedly active when mixed to produce an oxidation-reduction system.

The oligodynamic action of metals is possibly a result of the solution of the metal to form salts. The destruction of bacteria in contact with or in proximity to a piece of metal is the basis of some methods of disinfection of water. Water may be sterilized by allowing it to seep through a layer of silver-coated sand, and water containing colloidal silver in amounts sufficiently small to defy chemical detection, by calculation

about 40 γ per ml., is markedly bactericidal. Such colloidal solutions are prepared by sputtering silver electrodes in water—the so-called catadyn process.

The other cations are less toxic than mercury and silver, but even sodium and potassium are toxic to bacteria in sufficiently high concentrations (ca. 2 molar). It is of some interest that the arrangement of cations in an order of their toxicity-mercury and silver at one end and sodium and potassium at the other, with others falling in order in between-corresponds closely to the Hofmeister series and the lyotropic series of Freundlich, in which cations are arranged in the order of their effects on physical properties of proteins, such as coagulation, solubility, and viscosity. The toxicity of cations as manifested in solutions containing a single salt may often be neutralized by the presence, in the proper proportion, of another cation. This phenomenon, known as the antagonistic effect of salts, has led to the concept and preparation of so-called balanced solutions such as Ringer's solution and Locke's solution. Salts not only modify or enhance the toxic qualities of other salts but also exert similar effects on disinfectant compounds of widely different constitution. Sodium chloride, for example, markedly enhances the germicidal qualities of phenol for anthrax spores when present in sufficiently high concentrations.

The part which anions play in the growth and destruction of bacteria is less well known. Some, particularly those containing sulfur, carbon, nitrogen, or oxygen, may serve as sources of these elements and of energy, but in appropriate concentrations many are toxic for bacteria.

Oxidizing agents. Other salts, such as potassium permanganate and the sodium and calcium salts of hypochlorous acid (HOCl), show marked bactericidal activity owing to their properties as oxidizing agents. Mol for mol, hypochlorous acid is one of the most powerful germicides known, and its calcium salt (commonly known as bleaching power) has a wide use in the treatment of private and small municipal water supplies. Hypochlorous acid reacts with organic compounds containing an amide group with the formation of compounds known as chloramines. These compounds show strong disinfectant properties which are apparently associated with the presence of the =NCl group. Two of these, chloramine-T and PHENOLS 187

dichloramine-T, were used with considerable success in the disinfection of deep wounds in World War I. Similar compounds such as the chlorates and perchlorates likewise are bactericidal. The high bactericidal activity of chlorine dioxide has been of considerable interest in connection with its use in the treatment of water supplies, since it is relatively unaffected by alkalinity but, unlike hypochlorite and chlorine, its residuals, largely organic chlorine compounds, are relatively inactive.8 The halogens, chlorine, bromine, and iodine, are also potent germicides but, in contrast to the heavy metals, in inverse order to their atomic weights. Liquid chlorine is widely used in the treatment of water supplies, and iodine in the form of its tincture is an efficient skin disinfectant. Bromine has been used occasionally as a disinfectant for swimming pool water.62 Fluorine compounds are seldom used as disinfectants, although sodium fluoride is toxic to some bacteria, presumably owing to its interference with their oxidative mechanisms. Both hydrogen peroxide and ozone are bactericidal, but the former is rapidly decomposed by tissue catalase and has little penetrating power when applied to wounds and abrasions.

Organic compounds. A variety of organic compounds have antimicrobial activity and are useful as antiseptics and disinfectants. The more important are certain metal-organic compounds, phenols and related compounds, soaps and alkyl sulfate detergents, quaternary ammonium compounds, and a few alcohols and ethers.

Metal-organic compounds. More or less successful attempts have been made to utilize the germicidal activity of mercury and silver through the preparation of organic compounds which, while having disinfectant properties, are not markedly toxic to body tissue. These include Metaphen, Merthiolate, Mercurochrome (dibrom-oxymercurifluorescein), Mercarbolide, Merphenyl nitrate, Mertoxol (acetoxymercuri-2-ethylhexyl-phenolsulfonic acid), and Meroxyl

(sodium salt of 2-4-dihydroxymercury benzophenone-2'-sulfonic acid), together with a series of silver-protein compounds such as Argyrol, Protargol, Argonin, and the like (1). The organic compounds of mercury are generally used as skin disinfectants, though they are of doubtful value, and, in dilute solution, as preservatives. Merthiolate, for example, in 1:10,000 concentration is an excellent and widely accepted preservative for antiserums and similar biologicals. The silver compounds have utility in the treatment of infections of the mucous membranes.

Phenols. Phenol and its derivatives are among the most useful of the antibacterial organic compounds. Phenol itself destroys the vegetative cells of bacteria rapidly, and spores more slowly, in 5 per cent aqueous solution, and its activity is not seriously reduced in the presence of organic matter. It is used as the standard of comparison for other disinfectants, particularly those of similar chemical structure.

The activity of phenol is enhanced by substitution in the ring. The methyl phenols, ortho, meta, and para cresols, and halogenated phenols have greater activity than the parent compound. Resorcinol, hydroxyphenol, however, is only mildly bactericidal. In general, the substitution of aliphatic side chains in both phenol and hydroxyphenol increases antibacterial activity in direct proportion to the length of the side chain, but solubility in water is decreased to limit the practical value of such compounds.⁹

The bisphenols have become the most useful of the phenolic disinfectants because of their relatively high bacteriostatic and fungistatic properties and relatively low toxicity. As indicated by the name, these compounds consist of two phenol rings attached carbon-to-carbon or through oxygen, sulfur, or alkalene, especially methylene, groups. The position of the linkage with respect to the phenolic hydroxyl group is an important factor, and the nomenclature of these compounds becomes involved be-

1.

Mercarbolide

cause the carbon numbered 1 may be that to which the hydroxyl group is attached or that at which the linkage occurs. When the compound is symmetrical either may be used, but only the latter nomenclature may be used when it is asymmetrical.

The most important of these compounds are orthohydroxydiphenyl and the chlorinated methylene and sulfur compounds. Orthohydroxydiphenyl is marketed as O'Syl and Lysol, the latter containing cresylic acid also. There are three common chlorophenes. The simplest is dichlorophene (G-4) in which the methylene linkage is ortho to the hydroxy groups and the chlorines in the para positions. Depending upon the system of nomenclature this compound is 2,2'-methylenebis[4-chlorophenol], chloro-2-hydroxyphenyl]methane, or 2,2'dihydroxy-5,5'dichlorophenylmethane. The other two are tetrachlorophene (G-5) containing two additional chlorines, one on each ring ortho to the hydroxyl group, and hexachlorophene (G-11). The thio bisphenols in common use are bithionol (G-5-S), which corresponds to tetrachlorophene in that it contains four chlorines, and G-11-S, which corresponds to hexachlorophene (2). Hexachlorophene has the unusual and useful property of retaining substantially all its antibacterial activity when incorporated into soaps. These compounds are relatively insoluble in water, but are soluble in dilute alkali and in many organic solvents.

These compounds differ from phenol in their activity in that, while they are somewhat more active than phenol under the conditions of the phenol coefficient test (see below), more prolonged exposure is required for maximal bactericidal activity, and they are bacteriostatic in high dilutions. Dichlorophene, for example, gives phenol coefficients of 20 to 40 against staphylococcus and 50 to 75 against the typhoid bacillus, and hexachlorophene 25 to 50 against the former test organism and 5 to 10 against the latter. Dichlorophene, however, inhibits the growth of staphylococcus in dilutions of 1:500,000, and hexachlorophene in dilutions as high as 1:2,500,000.

Detergents. The surface-active detergent substances fall into three groups (3). The anionic compounds are soaps, sodium and potassium salts of higher fatty acids, and the alkyl sulfates such as sodium lauryl sulfate. The quaternary ammonium compounds are cationic substances, and the nonionic group includes polyethers and polyglycerol esters. Certain of these substances have antibacterial activity.

The antimicrobial activity of soaps is limited, and they cannot be regarded as

3.
$$\begin{bmatrix} R - C - O \end{bmatrix} = \begin{bmatrix} O \\ R - O - S - O \end{bmatrix} = \begin{bmatrix} O \\ R - S - O \end{bmatrix}$$
anionic
$$\begin{bmatrix} R_1 \\ R - N - R_2 \\ R_3 \end{bmatrix} + \begin{bmatrix} H & H & H \\ R - C - O - C - C - H \\ O & H & OH & OH \end{bmatrix}$$
cationic
nonionic

Surface-active (detergent) substances

DETERGENTS 189

effective antiseptics or disinfectants. They are useful agents for the mechanical removal of bacteria from the skin by emulsification of lipoidal secretions in which microorganisms are embedded. Thus the number of bacteria present on the skin is markedly, but temporarily, reduced by washing with soap. An appreciable antibacterial activity may be given soaps by combining them with disinfectant substances, commonly coal tar products such as cresols and related compounds, but, in general, the activity of the incorporated substance is reduced in admixture with soap. Hexachlorophene is, as noted above, a significant exception to this general rule. The use of hexachlorophenecontaining soaps results not only in an immediate reduction of the numbers of bacteria present on the skin, but the bacteriostatic activity of the residual hexachlorophene significantly inhibits the growth of bacteria on the skin.

Some of the alkyl sulfates have more antibacterial activity than soaps, inhibiting growth in relatively high (0.1 per cent) concentrations. Those which are active are markedly selective, affecting gram-positive bacteria but not gram-negative bacteria.

The quaternary ammonium compounds, often called "quats," are a group of amines containing pentavalent nitrogen and may be regarded as derivatives of ammonium chloride in which various radicals are substituted for the hydrogens. Ordinarily one is a long-chain (C_8 to C_{18}) alkyl group,

and the others smaller alkyl groups, phenyl groups, etc. Very many, perhaps a thousand, of these compounds have been synthesized, and several, including Zephiran (alkyldimethylbenzylammonium chloride), Ceepryn (cetylpyridinium chloride), Phemerol (diisobutylphenoxyethoxyethyldimethylbenzyl ammonium chloride), and Diaparene (diisobutylcresoxyethoxyethyldimethylbenzyl ammonium chloride) have gained general acceptance (4).

These substances are equally effective against gram-positive and gram-negative bacteria. They vary somewhat in activity. Zephiran, for example, is bactericidal to staphylococcus in dilutions as high as about 1:40,000 and bacteriostatic in dilutions as high as 1:200,000. The phenol coefficient of this compound has been found to range from 150 to 300 at room temperature against both staphylococcus and the typhoid bacillus, but the applicability of the phenol coefficient method to the assay of the activity of compounds of this kind is open to question.

There is a marked incompatibility between anionic and cationic detergents in that in mixture the antibacterial activity disappears. The nonionic detergents do not have this effect and, for example, quaternary ammonium compounds may be mixed with nonionic detergents having good solubilizing activity to give an antibacterial cleansing agent.

Alcohol and ethers. Ethyl alcohol and ethyl ether, often used as skin disinfectants,

4.

BATAN TRADAPAN TANAH J

$$\begin{bmatrix} CH_3 \\ C_nH_{2n}-N-CH_2 \\ CH_3 \end{bmatrix} CI \begin{bmatrix} N-C_{16}H_{33} \\ Ceepryn \end{bmatrix} CI$$

$$\begin{bmatrix} CH_3 \\ CB_1 \\ CH_3 \\ CH_3 \end{bmatrix} CO-C_2H_4-O-C_2H_4-N-CH_2 \\ CH_3 \\ Diaparene\ chloride \end{bmatrix} CH_3$$

$$\begin{bmatrix} CH_3 \\ CH_3 \\ CH_3 \\ CH_3 \end{bmatrix} CC$$

are not very good germicides. Their effectiveness probably lies in the solution of the lipoidal secretions of the skin and consequent mechanical removal of microorganisms. Absolute alcohol has little or no germicidal activity. The bactericidal activity of alcohol-water solutions increases with the addition of water, but 50 per cent alcohol and less has little activity; 70 per cent is the concentration usually used for skin disinfection. Absolute propyl and isopropyl alcohols are likewise ineffective but show activity in aqueous solution, while absolute methyl alcohol is said to be bactericidal.

Gaseous disinfectants. 11, 40 The use of bactericidal gases for the disinfection of rooms, dwellings, and the like (fumigation or terminal disinfection) has declined markedly in recent years with no coincident increase in the prevalence of infectious disease. The commonly used gas, sulfur dioxide (generated by burning flowers of sulfur), is probably not bactericidal as a gas but is bactericidal in aqueous solution; it is effective, therefore, only in the presence of adequate amounts of moisture (a relative humidity of 60 per cent or higher). Other gases such as hydrogen cyanide have little or no effect on bacteria. Although the value of terminal disinfection is open to serious question, that of disinfestation is well established and the gases, hydrocyanic acid in particular, are widely used for the destruction of rats aboard ship, and like purposes.

Alkylating agents. Certain of these have been found to be highly effective bactericides in gaseous form, both on solid objects and solutions, and on bacteria suspended in air. These include ethylene oxide, propylene oxide, ethylene amine, methyl bromide, and formaldehyde. These substances have the unusual property of being relatively more effective in the destruction of bacterial spores than the usual disinfectants, and their activity seems to be irreversible and bactericidal.

Formaldehyde has been used for many years, but is often unsatisfactory because it penetrates poorly and requires a relatively high humidity. It has been used more recently in combination with low temperature steam-vacuum sterilization for textiles and similar materials.³

Ethylene oxide is a more recent development and is similarly highly effective. It forms an explosive mixture with air, but the attendant danger is prevented by mixing with 7 to 10 volumes of carbon dioxide; the mixture is commonly called *carboxide*. It may be applied to fabrics or equipment of various kinds under pressure; the objects to be sterilized are treated in the dressing sterilizer type of autoclave which is evacuated to 14 pounds, and ethylene oxide run in to 20 to 23 pounds pressure and left overnight. Relative humidity is an important factor,²⁷ and it has been reported that ethylene oxide is more effective at higher temperatures.¹⁹

It is of some interest too that culture mediums can be so sterilized, with apparently no residual antibacterial activity remaining. This method of sterilization is particularly applicable to plastic disposable petri dishes and test tubes which cannot be sterilized by the conventional heating methods.

Another alkylating agent which has been of particular interest is β-propiolactone. 15, 38 Unlike ethylene oxide, it is not inflammable, but requires a high humidity (80 per cent) to be effective, and has only limited penetrating power so that it functions most effectively as a surface disinfectant. It is highly bactericidal and virucidal in concentrations of 1 to 5 mg. per liter and is considered to be about 25 times more effective than formaldehyde. While lacking the general utility of ethylene oxide, it is especially effective in the decontamination of enclosed spaces such as rooms and buildings. 12, 90 and may also be used for the sterilization of heat-labile materials.34 It is viewed with reservation by some because it has been found to be carcinogenic in the mouse.

Aerosols. An aerosol may be defined as a finely dispersed substance in air, and aerosols are of interest in connection with the destruction of air-borne microorganisms. Suitable substances must not only have antimicrobial activity, but also be sufficiently nontoxic and nonirritating that they may be inhaled by man and other animals for reasonable periods. The most commonly used compounds are glycols, especially triethylene glycol.

The glycol is relatively nontoxic and the vapor may be breathed with impunity, but bactericidal concentrations of 50 per cent or higher are attained in contact with suspended microorganisms. The rate of destruction of air-borne bacteria is in agreement with the assumption that the glycol mole-

cules in the vapor state condense on the bacteria-containing droplets, and efficacy is associated with a low vapor pressure, high hygroscopicity, and, of course, toxicity for the bacterial cell substance.^{57, 74, 75}

While pathogenic microorganisms such as pneumococcus, streptococcus, and influenza virus are killed or inactivated rapidly under experimental conditions, in general aerosols have given disappointing results when used for practical control of air-borne infection.

Dyes. The dyes are widely used in bacteriology both for staining purposes and as indicators. In addition, many of them show a marked bacteriostatic and bactericidal activity which is often specific in that it is manifested against one organism and not another. The incorporation of an appropriate dye in a medium will render it selective; i.e., it will favor the growth of some species of bacteria and inhibit that of others. In general, this specificity is correlated with the Gram reaction; the gram-negative organisms are, for the most part, much less sensitive to dyes than are the gram-positive species. The activity of these compounds is affected by pH, the toxicity of the acid dyes increasing with acidity and that of the basic dyes increasing with alkalinity.

Many of the dyes such as the thiazins, oxazins, and azo dyes are not particularly toxic for bacteria, dilutions of 1:1000 or less being required to inhibit growth. A number of the triphenyl methane dves are. on the other hand, inhibitory in high dilutions. Malachite green, for example, inhibits the growth of B. subtilis in dilutions 1:4,000,000 and staphylococci in 1:1,000,000, while higher concentrations, i.e., 1:30,000 to 1:40,000, are required to inhibit the colon and typhoid bacilli. Victoria green, a dichlor derivative of malachite green, is bacteriostatic to about the same degree. Brilliant green is active in even higher dilutions, inhibiting B. subtilis in a dilution of 1:15,000,000 and staphylococci in 1:4,000,000, and the typhoid and colon bacilli in 1:500,000.

The bacteriostatic properties of the triamino-triphenyl methane dyes, the so-called rosanilins, are apparently associated with the substitution of alkyl groups in the amido side chains. Basic fuchsin, a mixture of the unsubstituted simple dyes rosanilin and pararosanilin, is relatively weakly bacteriostatic, dilutions of 1:500,000 being required

to inhibit the growth of B. subtilis. Acid fuchsin, a mixture of various sulfonated derivatives of basic fuchsin, is likewise only weakly inhibitory and was formerly widely used in mediums as an acid indicator under the name of Andrade's indicator. On the other hand, methyl violet* a mixture of tetra-, penta-, and hexamethyl pararosanilin, is markedly bacteriostatic and completely inhibits the growth of bacteria such as staphylococci and diphtheria bacilli in dilutions of 1:1,000,000 to 1:5,000,000. Approximately 150 times as much dye is necessary to suppress growth of the less sensitive gram-negative bacteria such as the colon and typhoid bacilli. There appears to be a correlation of bactericidal activity and basicity of these compounds.

The acridine dyes, acriflavine and trypaflavine, have been of particular interest because of their therapeutic significance. The former is actively bacteriostatic in dilutions as high as 1:3,000,000, and the latter inhibitive to staphylococci and similar organisms in 1:2,000,000 and to the relatively fragile gonococcus in dilutions of 1:10,000,000 to 1:50,000,000. The gonococcus is killed by exposure to the dye in dilutions of 1:80,000 to 1:400,000 within two or three minutes.

The mechanisms of bactericidal action. The ways in which antimicrobial substances affect the cell so as to produce changes in its organization resulting in growth inhibition or death are varied and, as might be expected, dissimilar effects on the cell give the same end result. A substance showing bactericidal properties produces its effects through an interruption of essential physiological processes of the bacterial cell. In some instances the effect is relatively nonspecific, as in the denaturation of constituent protein, and in others highly specific in that some particular metabolic reaction is affected. There is some tendency to differentiate the former as a general protoplasmic poisoning, but this is more a matter of convenience than an expression of fundamental difference. A continuous series of degrees of specificity, so to speak, may be set up through which the general toxic effects grade imperceptibly into the inhibition of a single enzyme system.

^{*}Gentian violet is a more or less impure mixture of methyl violet and dextrin. Crystal violet, hexamethyl-p-rosanilin, is one of the constituents of methyl violet.

Thus, a general oxidation of cell substance by oxidizing agents, a saturation of thiol groups of constituent proteins with heavy metals, or a generalized denaturation of protein by, for example, substitution of halogens such as iodine in the ring structures of aromatic amino acids, obviously alters the organization of the cell on a macromolecular level to such an extent that normal metabolism cannot continue. Bactericidal substances of the phenol group apparently act in part in this way, in that phenol is surface-active and by orientation of the hydroxy group reacts with free amino groups of the cell proteins.

Or the effect may be somewhat more localized as in the case of the basic dves which react with nucleoprotein and whose action, at least that of acriflavine, is antagonized by nucleotides. Similarly, the surfaceactive detergents are antagonized by lipids. e.g., the anionic detergents by cephalin, and there is some reason to believe that they alter the permeability of the cell wall to such an extent that the cell dies. Phenols and other substances act similarly, and in general the antimicrobial effect of such agents is a net one, with leakage of cell contents due to permeability effects, possibly general protein denaturation, and adverse effects on the availability of coenzymes, activating ions, etc.

Generally speaking, the loss of enzyme activity is the most important single factor, 92 and even antimicrobial substances that appear to be general protoplasmic poisons often show a specificity of action. There is evidence that chlorine, for example, first affects a triosephosphate dehydrogenase Sulfhydryl-containing appear to be the most susceptible to the action of bactericidal substances, and poisoning by heavy metals, oxidizing agents, etc., occurs with some facility. The alkylating agents such as ethylene oxide act by direct alkylation, and the thiol group is probably the most vulnerable point in the cell organization. Azide markedly inhibits esterification of inorganic phosphate, and it has been suggested that it reacts with the acyl phosphate of diphosphoglycerate. The acyl azide is spontaneously decomposed and thus drains the energy-rich phosphate formed.

Often antimicrobial activity is paralleled by an inhibition of oxygen uptake, indicating a poisoning of the respiratory enzymes, and measurement of such inhibition has been suggested as a method of assaying bactericidal activity. This approach is not always valid, however, for some substances such as dinitrophenol stimulate oxygen uptake and prevent the assimilation of carbon; it inhibits assimilation by uncoupling phosphorylation and draining away the energy stored in energy-rich phosphate bonds. It will be clear that while information as to the precise point of attack of antimicrobial substances on cell organization and function is by no means complete, a variety of mechanisms are operative.

Reversal of antibacterial activity. croorganisms exposed to the action of a number of antimicrobial substances and apparently killed may be "revived" by neutralization of the activity within a short time after exposure. The revival of bacteria and viruses such as influenza virus, presumably dead after treatment with mercuric chloride, by treatment with hydrogen sulfide or thiols such as thioglycollic acid, glutathione, and the like is well established. Similarly, bacteria apparently killed with hypochlorite may be revived by inactivating the chlorine with sodium thiosulfate, and the use of thiosulfate solutions for dilution in titration of bactericidal activity of the hypochlorites to neutralize bacteriostatic concentrations carried over into subcultures is a common procedure. Bacteria treated with phenol survive if the phenol is removed by adsorption on charcoal or treatment with ferric chloride. The effects of treatment with dyes such as acriflavine or with cationic detergents may be neutralized with survival of the bacteria by high molecular weight anions or, in the case of dyes, more simply by shifting the pH so that the dye-protoplasm complex is no longer unionized, provided that the shift is within physiological limits.

In the case of bactericidal concentrations of the compound, it is not clear how far the bactericidal action can be allowed to go and yet permit revival. In many instances it is probable that it has not gone far, perhaps little beyond a preliminary sorption. When the compound is in bacteriostatic concentration only, of course, the cells remain alive for relatively long periods and multiply as soon as the inhibition is removed by neutralization or simple dilution. The significance of reversal of the antimicrobial effect lies in the inferences that may be drawn as to the nature of the antimicrobial activity.

Thespecificity of disinfectants. The marked specificity of the bacteriostatic and bactericidal activity of the dyes has been referred to above. The property of differential toxicity is not confined to these compounds alone, however, but is exhibited to some degree by many of the bactericidal chemicals. The hypochlorites, for example, while powerful germicides for most bacteria, have little effect on the tubercle bacillus, and it is of practical significance also that poliovirus persists in treated waters containing sufficient chlorine to destroy pathogenic bacterial contaminants.⁵³ general, the salts of the heavy metals are the least specific in their action and dyes the most, with other compounds lying between these two extremes. Certain slow oxidizing agents such as potassium dichromate exert a selective bacteriostatic effect on gram-negative bacteria, and iodine is more efficacious against these microorganisms. Similarly, among the long-chain aliphatic bases, the less strongly basic amines act on gram-positive bacteria for the most part, whereas the gram-negative organisms are more susceptible to the action of the stronger bases such as guanidines and quaternary amines. In general, the viruses are considerably more resistant to the activity of quaternary ammonium compounds than are bacteria. These compounds fail, for example, to inactivate vaccinia virus in dilutions as low as 1:1000 although they are bactericidal in many thousand-fold dilution.

influencing disinfection. The Factors process of disinfection or bacterial death is often, in part at least, a chemical reaction and is, therefore, subject to a variety of influences which affect the velocity of such reactions. The most important of these influences is the concentration of the reacting substances, i.e., the concentration of disinfectant and the numbers of bacteria present. The effective concentration of disinfectant is, in turn, dependent upon two other factors: first, the presence of moisture. which makes possible coagulation by heat and ionization of the bactericidal salts, and acts as a solvent and suspending medium in which there may be intimate contact between the disinfectant and the microorganism; second, the presence of extraneous organic matter.

Many chemical disinfectants act through

a combination with the protein of the cell and, if extraneous organic matter is present, will, of course, react with this inert material, thereby reducing the effective concentration. Disinfectants vary widely in the degree to which their bactericidal properties are affected by organic matter. The salts of the heavy metals are rapidly precipitated by organic material, while compounds such as phenol and the cresols are only slightly affected. The rate of destruction by heat is also affected by the presence of organic matter-organisms embedded in a mass of fecal material, for example, are protected from heat for a short time. The process of disinfection by germicides or by heat is influenced by temperature, the velocity of the reaction increasing with rise in temperature.

The pH likewise influences the rate of bacterial destruction not only by heat but by many chemical compounds, the velocity, in general, being least at neutrality and increasing with increase in acidity or alkalinity. A number of other factors, such as the presence of salts, ¹⁰⁸ affect the rate of disinfection but generally not sufficiently to be of practical importance.

From the practical point of view, the time of exposure of bacteria to a given disinfectant is of considerable significance and bears an inverse relation to the rapidity of killing. The time allowed for the destruction of bacteria is determined not only by the factors discussed above but also by the kind of bacteria that are to be killed. In certain cases the specificity of a disinfectant may be so marked that it must be taken into consideration. For example, the relative atoxicity of hypochlorite for the tubercle bacillus referred to above precludes its use in the disinfection of tuberculous sputum. Bacterial spores are much more resistant to heat and chemical disinfectants than are the vegetative cells, and considerably more time must be allowed for their destruction. The vegetative cells of some bacteria may be somewhat more resistant than those of others, but, for the most part, such differences are too small to be of practical significance.

The dynamics of disinfection.^{37, 98} Quantitative studies on the rate at which microorganisms are killed by lethal agents have indicated that in many instances the organisms die at a logarithmic rate; *i.e.*, if the logarithms of the numbers of viable organ-

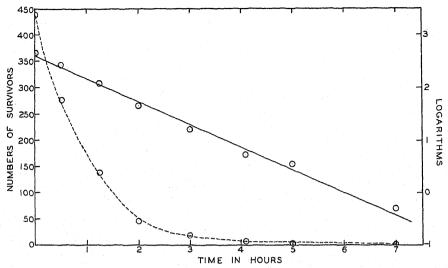


Figure 47. Death rate of anthrax spores treated with 5 per cent phenol. The dotted line is the arithmetic plot and the solid line the logarithmic plot. The negative logarithm was obtained by taking samples of several times the unit volume. (After Chick.)

isms are plotted against time, the points tend to fall on a straight line. This phenomenon has been observed in the death of both spores and vegetative cells under the influence of chemical disinfectants or moist heat and also occurs in the death of bacteria in old cultures. The velocity of the reaction, the slope of such a line, depends upon the concentration and kind of disinfectant, the

nature of the organisms—whether spores or vegetative cells—and other factors which influence the process of disinfection. This logarithmic rate likewise describes the course of a monomolecular reaction, and this fact has led some to conclude that bacterial death is a monomolecular chemical reaction. Although the killing of bacteria by some disinfectants is undoubtedly a

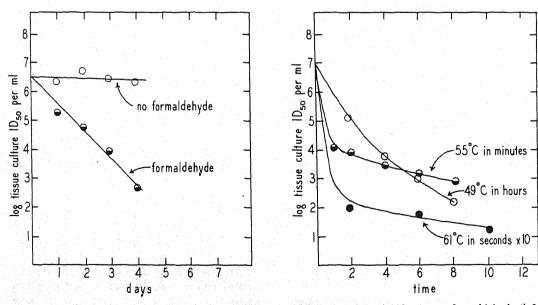


Figure 48. The inactivation of tissue culture foot-and-mouth disease virus by 0.009 per cent formaldehyde (*left*) and by heat (*right*). The formaldehyde inactivation is exponential over the indicated range, but deviation from an exponential rate during heat inactivation is marked, with increased heat stability at low survival levels. (Data from Bachrach, Breese, Callis, Hess, and Patty.)

chemical process, as for example the precipitation of constituent protein as proteinates of heavy metals, the evidence does not justify the conclusion that the reaction is monomolecular. The fallacy is the old one of confusion of correlation and causality under the slightly different guise of description and mechanism. Experimental evidence has indicated that while such semilogarithmic plots may often be fitted with a straight line, others are best fitted by sigmoid curves, the death rate being more rapid in the beginning in some cases and more rapid at the end in others or even highly irregular.

One of the more important determining factors is a graded biological resistance of the cells in the bacterial population, an explanation that is supported by general biological considerations. Undoubtedly the phenomenon of bacterial death is a complex of interrelated factors whose admixture with the mechanics of the mass law results in the parameters determined by mathematical means. Of practical importance, however, is the fact that in disinfection by chemicals and by heat there is a minority of cells, possibly more resistant, that survive long after the majority have perished and that must be destroyed in order to obtain complete sterilization. The determination of antiviral activity poses special technical problems because of the necessity for assay of viable agent by infectivity titrations,⁴⁷ and the effects of auto-interference when a large portion of the agent has been inactivated toward the end point (Chap. Four).

In any case, it is clearly not possible to extrapolate the exponential death rate to zero and assume that the time of exposure so indicated insures sterility; such an assumption resulted, for example, in incomplete inactivation of poliovirus by formalin in the preparation of vaccines. 106

The standardization of disinfectants. The relative bactericidal efficiency of the chemical disinfectants is a point of considerable practical importance. The value of a quantitative method for the determination of the killing power of germicides was recognized early in the development of bacteriology, and experimental investigation led to the establishing of a standardized technique which made possible the determination of the bactericidal power of a given chemical compound relative to that of phenol. The numerical value so determined is termed the phenol coefficient and is presumed to

indicate whether, and to approximately what extent, the unknown is a better or poorer germicide than phenol. Later methods of standardization have grown out of this procedure. The phenol coefficient of a disinfectant used against the typhoid bacillus is calculated as follows:

Divide the greatest dilution of the disinfectant capable of killing Salmonella typhi in 10 minutes but not in five minutes by the phenol dilution which so kills and divide these figures one into another. In order not to convey a false idea of the accuracy of the method the coefficient is calculated to the nearest 0.1 point if under 1, to the nearest 0.2 point if between 1 and 5, to the nearest 0.5 point if between 5 and 10, and to the nearest 1.0 point if between 10 and 20. The conditions regarded as standardized in the United States, the FDA phenol coefficient, have been defined by the Food and Drug Administration.

The effect of extraneous organic matter on the bactericidal power of a disinfectant is commonly taken into consideration by carrying out the test with and without added organic matter. Three per cent of dried fecal matter or dried yeast may be added to the bacterial suspension or the organisms may be suspended in 50 per cent serum. It is important to recognize that bacteria differ considerably in their resistance to phenol, staphylococci, for example, being much

Determination of the Phenol Coefficient*

DAGINATION AND		TIMI	TIME IN MINUTES						
DISINFECTANT	DILU- TION	5	10	15					
Unknown	1:300	0	0	0					
	1:325	+	0	0					
	1:350	+	0	0					
	1:375	+	+ 1	0					
	1:400	+	+	+					
Phenol	1:90	+	0 0	0					
	1:100	+	+	+					

The test is satisfactory only when the resistance of the test organism, here Sal. typhi, gives one or another of the following reactions:

$$\frac{1:90 + \text{or } 0 + \text{or } 0}{1:100} + \frac{0}{0}$$

$$\frac{350}{90} = 3.89 = 3.9$$

^{*}From Stuart, in Reddish, G. F.: Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization. 2nd ed. Philadelphia, Lea & Febiger, 1957.

more resistant than the typhoid bacillus, so that in strict accuracy it is necessary to specify "typhoid phenol coefficient," "pneumococcus phenol coefficient," etc.

The phenol coefficient criterion of antimicrobial activity has many defects, including failure to evaluate concentration effects, temperature coefficients, etc. In addition, the validity of a phenol coefficient determined for a nonphenolic disinfectant is open to serious question. In consequence, many variations of the procedure have been used. For example, the germicidal equivalent concentration test has been developed for the evaluation of the lethal activity of substances such as hypochlorites, in which concentrations giving the same killing pattern as those of a reference compound are determined. This kind of test is illustrated in the accompanying tabled example; here the activity of the unknown disinfectant in a concentration of 10 ppm is equivalent to that of the reference standard, NaOCl, in a concentration of 50 ppm.

Assay of the antimicrobial activity 103 of detergents is difficult because of the tendency of the test bacteria to become adsorbed to the walls of the test tube as a consequence of altered charge in the presence of the disinfectant. So-called use dilution tests have been employed for these substances in which a suspension of the test microorganism is dried on the surface of a carrier such as glass rods or rings, immersed in the disinfectant for varying periods, and subsequently cultured. Such methods are not new but were used many years ago by Koch and others who dried anthrax spores on silk threads, glass beads, etc., for testing bactericidal activity.

All of these methods of assay suffer from the fundamental defect of dependence upon a sterility end point, an end point which studies of the dynamics of the process of disinfection have shown to be highly questionable.

It is generally agreed that the rate of killing, *i.e.*, the reaction velocity constant, is a much more accurate measure than any value whose derivation depends upon a sterility end point. The relation between concentration of disinfectant and time required for killing is an exponential one, and the reaction velocity constant, k, is given by the expression:

 $k = C^n t$

where C is the concentration, n a constant characteristic for each disinfectant, and t is time. The temperature coefficient may, of course, be determined experimentally for each disinfectant. It will be clear that this kind of characterization of the bactericidal activity of a given compound is much more informative than the usual type of phenol coefficient, no matter how precisely the conditions of the test are defined. It is not, however, generally used for routine work.⁵⁰

The practical value of a disinfectant is not always indicated by tests made under the controlled conditions of the laboratory. Hydrogen peroxide, for example, may give a quite respectable phenol coefficient but, when applied to an abrasion, is so rapidly decomposed under the influence of tissue catalase that its germicidal powers are almost immediately exhausted. This has led to various kinds of infection prevention tests which have necessarily included the

The Germicidal Equivalent Concentration Test*

					su	BCUL	TURE	SERII	Es†			
DISINFECTANT	CONCENTRATION (in ppm)	1	2	3	4	5		6	7	8	9	10
Unknown	10	0	0	+	+	+		+	+	+	+	+
	20	0	0	0	0	4		+ '	+	+	+	+
	25	0	0	0	0	C		+	+	+	+	+
NaOCl control	50	0	0	+	+	-4		+	+	+	+	+
그리다의 취하하다는 남자는 글로	100	0	0	0	+	4		+	+	+	+	+
	200	0	0	0	0	()	0	+	+ , ,	+	+

^{*}From Stuart, in Reddish, G. F.: Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization. 2nd ed. Philadelphia, Lea & Febiger, 1957.

[†]The time interval may vary from 30 seconds to two minutes.

matter of the toxicity of the disinfectant for tissue cells.

A given disinfectant may be so highly toxic for tissue cells that it has no practical value. Salle and his associates84 have proposed a "toxicity index" which takes into consideration not only the germicidal activity of a compound but its toxicity for tissue as well. For example, Witlin¹¹⁰ determined the concentration of bactericidal substances lethal for the chick embryo and calculated a toxicity index by using the concentration of the bactericide in grams per milliliter killing the test organism, Staphylococcus aureus, in 10 minutes but not in five as the numerator, and the chick embryo MLD in grams for the denominator. Representative values were: phenol (1:20)-1.18; $HgCl_2$ (1:1000) – 0.62; tincture of iodine-0.044; sodium hypochlorite-1.39; Mercurochrome (1:50)-13.3; and values

ranging as high as 9.1 for various organic mercurials. Spaulding and Bondi⁸⁷ have developed an infection-prevention-toxicity test in which the tip of a mouse tail is contaminated with bacteria, the tail dipped into the solution of disinfectant, and then the tip is removed and placed in the peritoneal cavity. The highest dilution of the disinfectant protecting 50 per cent of the mice is taken as the numerator, and the greatest concentration allowing survival (from poisoning) of the test animal is taken as the denominator, to give a ratio or infectionprevention-toxicity index. Such toxicity indexes are of considerable practical importance in the case of skin disinfectants.

The whole question of the evaluation of disinfectants is beset with many difficulties, both theoretical and practical, and to date remains an open one to which no entirely satisfactory answer has been supplied.

The Chemotherapeutic Drugs 23

Antibacterial activity as described previously is characterized by its manifestation in vitro, i.e., against the living microorganism but outside the body of the host in the case of the pathogenic microorganisms, and by the end point of bacterial death, a criterion of bactericidal activity. The feasibility of using antibacterial substances in vivo as chemotherapeutic agents is dependent primarily upon the phenomenon of specificity of action of such substances, specificity as regards selective action on the microorganism without significant harmful effects on the host. This idea is by no means new; it was the basis of Ehrlich's search for a "magic bullet," a compound strongly germicidal for a given microorganism yet sufficiently nontoxic that it could be injected in suitable amount to give effective concentrations in the tissues. His work, originally directed toward the therapy of African sleeping sickness of trypanosome etiology, was an attempt to retain the antimicrobial activity of arsenic compounds and at the same time reduce toxicity for the host, and culminated in the synthesis of salvarsan. The original work has, of course, been greatly extended and amplified both by Ehrlich and other workers through the preparation of other arsenicals, antimony compounds, and so forth.

Prior to 1940 the approach to chemotherapy of infectious diseases was fundamentally the same, a trial-and-error search for antimicrobial activity in vivo, followed by determination of the active portion of the molecule, and retention of this activity with coincident reduction of toxicity to the host by the synthesis and testing of a great number of related compounds. For example, the small but significant antimalarial activity of the substituted thionine, methylene blue, was exploited by the German workers in the development of Atabrine. A general practice, then, has been the routine testing of synthetic organic chemicals, especially dyestuffs and dye intermediates, for antimicrobial activity in vivo. This procedure led Domagk in 1935 to the observation of the marked chemotherapeutic activity of the dye Prontosil in experimental streptococcus infections. Routine procedure led to identification of the active portion of the molecule, p-aminobenzene sulfonamide (sulfanilamide), and the synthesis of a great number of related compounds known as the sulfonamides. While of great practical significance, in that these compounds were the first found to be effective in bacterial infections, the discovery and study of the sulfonamides marks a great step forward in that (1) there is now available for the first time a rational theory of chemotherapy (see below) and (2) it is now clear that effective chemotherapeutic agents are usually bacteriostatic rather than bactericidal.

As implied earlier, the differentiation of bactericidal and bacteriostatic activity is essentially one of convenience based on the concentration differential of the active substance, i.e., when the difference between the two end points (growth inhibition and death) is great, the compound is regarded as primarily bacteriostatic, but when it is very small the bacteriostatic zone is so limited as to appear unimportant and the term bactericidal is usually applied. Compounds that are practical chemotherapeutic agents are, like the triphenylmethane dyes, bacteriostatic substances.

The chemotherapeutic activity of a substance employed in bacteriostatic concentrations rests in large part upon the host facet of the host-parasite relationship. Upon invasion of a susceptible host with a pathogenic microorganism, a series of mechanisms grouped under the head of resistance or natural immunity (Chap. Nine) become functional. The relationship between the host and the microorganism, then, is a dynamic one which is affected by lesser factors influencing one or the other. Antitoxin therapy, for example, reinforces the immune response of the host, tipping the balance against the microorganism. Similarly, the administration of a substance that does not interfere to a significant extent with the mechanisms of natural immunity, but does prevent the microorganism from proliferating within the tissues, also tips the balance against the microorganism to allow its destruction and elimination by the host. Formerly the role of host defense was not fully appreciated, and the significance of the failure to take this factor into account is underscored by the fact that sulfonamide compounds were investigated by one of the outstanding research laboratories as early as 1912 and discarded because they showed little bactericidal activity.

At the present time a number of highly effective chemotherapeutic agents are known. They may be divided into two groups: those that were originally synthetic organic chemicals and those that are derived from living organisms. The latter may be divided again into two subgroups: those substances that are formed by certain species of bacteria and those that are formed

by actinomycetes and molds. The structures of some of the naturally occurring substances are known, and one is prepared on a commercial scale by synthesis. All are characterized by being primarily bacteriostatic and by a specificity of action manifested not only by a lack of toxicity for the host, but also by differential activity with respect to species, and sometimes strains, of microorganisms.

SYNTHETIC COMPOUNDS

The synthetic chemotherapeutic agents, with the exception of the sulfonamides, are used in the therapy of diseases produced by the animal parasites and in the treatment of tuberculosis and leprosy. The first group is considered elsewhere (Chap. Thirty-four). The chemotherapeutic agents having greater or lesser efficiency in the treatment of infections with the acid-fast bacilli fall into four groups, viz., the sulfones, the aminohydroxybenzoic acids, the thiosemicarbazones, and the derivatives of pyridine carboxylic acid.

The sulfones. The antituberculous activity of the parent compound of the sulfone group, 4,4'-diaminodiphenylsulfone (DDS, DADPS, Dapsone), was reported by French workers in 1940. Various disubstitution products, Promin (sodium 4,4-diamino-diphenyl sulfone-N, N' didextrose sulfon-Promacetin (sodium 4,4'-diaminodiphenylsulfone-2-acetylsulfonamide), Diasone (disodium formaldehyde sulfoxalate diaminodiphenyl sulfone), and Sulphetrone $[\gamma$ -phenyl-N-propylamino] (4.4'-bis)phenylsulfonetetrasodium sulfonate) have been prepared in an effort to reduce toxicity and increase solubility and activity. None has been particularly successful, possibly in part because it is probable that the active derivatives are hydrolyzed to DDS in vivo and the activity is attributable to that of the parent compound. In general, the activity and toxicity of the sulfones are such that they are not highly effective chemotherapeutic agents and are not generally used in the treatment of tuberculosis since better drugs are available. However, these compounds are useful in the treatment of leprosy.

Aminohydroxybenzoic acids. Of these compounds, p-aminosalicylic acid (PAS) is the best known. Its activity against the

tubercle bacillus was reported in 1944, based on the observation made three years earlier that the oxygen uptake of the tubercle bacillus is increased in the presence of salicylates and benzoates. It is relatively nontoxic and is currently most useful when administered together with streptomycin, since it markedly reduces the tendency of tubercle bacilli to become resistant to the antibiotic (Chap. Thirty-one).

Many derivatives have, of course, been prepared, but of these only one, phenyl p-aminosalicylate (FR 7), first described in 1951, shows greater activity than PASabout ten-fold when given subcutaneously.

Thiosemicarbazones. These compounds, described as possible chemotherapeutic agents in 1946, are more active against the tubercle bacillus than are the sulfones and PAS. The parent compound (TB I) is not particularly active, but p-acetaminobenzaldehyde thiosemicarbazone (Tibione, TB 1/698, Conteben, Thiacetazone, Amithiozone) has considerable activity and has been widely used in Europe, where it has been found reasonably effective in most forms of tuberculosis. It is, however, toxic (gastrointestinal disturbances, anemia, liver, and kidney damage), and the incidence of severe side reactions is high. Many derivatives of this structure have been prepared, most of them involving alteration in the character and position of the subordinate grouping on the benzene ring. Of these, only the p-ethylsulfonyl compound (TB III) appears promising. Of the three isomers of the pyridine analogue of Tibione, the β and γ isomers have shown promise in experimental tuberculosis.

Certain of the thiosemicarbazones have been found to have activity against the poxviruses. The compound N-methylisatin β-thiosemicarbazone (methisazine, boran) has been of particular interest, since it has been found to be prophylactic, though not therapeutic, for smallpox in man, as well as antiviral in tissue cultures and prophylactic in experimental vaccinia.83, 104 The mechanism of the antiviral activity is quite uncertain; it apparently does not affect the synthesis of viral DNA or protein, but interferes with a step required for maturation, for the virus particles produced are noninfective.

Pyridine carboxylic acid compounds. The tuberculostatic activity of nicotinamide was reported in 1945; the activity is not, as stated by some workers, a function of

5.

isonicotinic acid hydrazide

1-isonicotinyl-2-isopropylhydrazine

its vitamin activity, since nicotinic acid has no antibacterial activity. Isonicotinic acid hydrazide (Rimifon, Nydrazid, Neoteben, INH) and 1-isonicotinyl-2-isopropylhydrazine (Marsilid) have been found to have marked activity, several-fold greater than streptomycin in experimental tuberculosis. Further study has shown that activity is not necessarily limited to immediate derivatives of nicotinamide, but may be associated with the pyridine structure, and pyrizinamide (Aldinamide), for example, has chemotherapeutic activity.

Nitrofurans.70 Furfural and related compounds have long been known to have antibacterial activity. The activity is greatly increased by substitution of a nitro group in the 5-position, and a great many derivatives have been prepared by substitution of various side-chains in the 2-position. The first of these to show promise as a chemotherapeutic agent was 5-nitro-2furaldehyde semicarbazone, or nitrofurazone (Furacin). Other nitrofurans include N-(5nitro-2-furfurylidene)-1-amino hydantoin, or nitrofurantoin (Furadantin), and N-(5-nitro-2-furfurylidene)-3-amino-3-oxalolidone, or furazolidone (Furoxone). As a group, these compounds have a broad antimicrobial spectrum, affecting certain fungi and protozoa as well as bacteria. Nitrofurazone has been used as a topical application in man in the therapy of burns, etc. Nitrofurantoin is excreted in the urine following oral administration and is an effective chemotherapeutic agent in urinary tract infections. Furazolidone remains in the intestinal tract following oral administration and is used in the treatment of enteric infections of bacterial etiology, such as Salmonella and Shigella, and is also a trichonomacide. These and other nitrofurans are also used in the therapy of various infectious diseases of domestic animals.

Sulfonamide compounds. 33, 100 cated above, p-aminobenzene sulfonamide was the first of the chemotherapeutic substances effective in bacterial infections other than the treponematoses that are susceptible to treatment with arsenicals. The term sulfonamide is generally taken to include the parent compound and its derivatives. Several thousand sulfonamides have been prepared, usually by substitution in the amido group attached to the sulfone radical, but some, such as Sulfathalidine and Sulfasuxidine, contain substitutions in the amino group attached directly to the benzene ring. These last are poorly absorbed from the intestinal tract and have considerable utility in some infections confined to the lumen of the bowel, such as bacillary dysentery. Of all the compounds prepared, only a few have been generally used. They are effective against microorganisms such as the streptococci, staphylococci, pneu-

6. NH2 NH₂ NH₂ ĊН ĊН H SO₂—NH₂ sulfanilamide sulfadiazine sulfapyridine NH_2 NH₂ NH_2 CH₃ CH₃ CH₃ CH₂ H sulfamethazine sulfamerazine Gantrisin, Gantrosan, sulfisoxazole, sulfafurazole

mococci, gonococcus, meningococcus, the plague bacillus, the dysentery bacilli, but are relatively ineffective against microorganisms of the Salmonella group, including the typhoid bacillus, rickettsiae, and others.

There appears to be little qualitative difference among these compounds so far as antibacterial activity is concerned, but they differ with respect to solubility, rates of absorption and excretion, and other factors. In general, they are only sparingly soluble in water, and solubility increases with alkalinity. On absorption a portion of the drug is inactivated by combination with plasma protein, and a portion acetylated to an inactive form in the liver, and both active and inactive drug are excreted in the urine. When the urine is acid and its volume low, the drug may accumulate in the kidney with resulting damage. The administration of mixtures of sulfonamides, such as triple sulfa containing sulfadiazine, sulfamethazine and sulfamerazine, has no direct therapeutic advantage, but reduces precipitation in the kidney since the solubility of each is independent of the presence of the others.

At the present time the sulfonamides are somewhat neglected as a consequence of the glamor of the antibiotics. While in many, but not all, instances they are less potent therapeutic agents, the sulfonamides will do just as much, more simply and cheaply, though possibly more slowly, than the antibiotics. They have the additional advantage that their administration produces less drastic changes in the normal flora and consequently less secondary infection with drug-resistant microorganisms.

THE ANTIMETABOLITE THEORY OF ANTIBACTERIAL ACTIVITY⁷⁶, 85, 111, 112

Competitive inhibition of enzyme reactions in which a structural analogue of the substrate competes with the substrate for the enzyme was described by Quastel and Wooldridge in 1928. They demonstrated the inhibition by malonic acid of the oxidation of succinic acid to fumaric acid catalyzed by succinic dehydrogenase in the presence of a hydrogen acceptor, and reversal of the inhibiting effect by excess succinate. Malonic acid differs from the

substrate in that it is a 3- instead of a 4-carbon dicarboxylic acid.

If such a reaction is an essential part of the metabolic processes of a bacterium, the inhibitor exhibits antibacterial activity. The immense practical significance of this was pointed up in the observation by Woods in 1940 that the antibacterial activity of sulfonamide is specifically inhibited by p-aminobenzoic acid, a compound that is required as a bacterial vitamin by a number of organisms, including Clostridium acetobutylicum, Acetobacter suboxydans, and some strains of the diphtheria bacillus. The close similarity in the structure of paminobenzoic acid and p-aminobenzene sulfonamides will be obvious from the structural formulas in 6. That p-aminobenzoic acid is required by some bacteria suggests that it is involved in some essential metabolic reaction, and it was suggested by Woods and by Fildes that the action of the sulfonamides on bacteria is the result of a competition of these compounds with an essential bacterial metabolite, p-aminobenzoic acid. This inhibitory effect of sulfonamides is reversible and can be antagonized by excess p-aminobenzoic acid. This has the practical value of allowing successful blood culture from individuals undergoing sulfonamide therapy by inclusion of p-aminobenzoic acid in the culture medium (5 mg. per 100 ml.).

The generalization follows that essential physiological reactions of the microorganisms may be inhibited by analogues of metabolites, and that such antimetabolites might be expected to show antibacterial activity. The plausibility of such a hypothesis is underscored by the essentially growth-inhibiting rather than lethal effects of chemotherapeutically effective substances on pathogenic microorganisms under physiologically tolerable conditions. This hypothesis assumes primary importance by providing, for the first time, a rational basis for the chemotherapy of infectious disease.

Enzyme inhibition. This theory is substantiated by impressive evidence. It has also been extended by the production of symptoms of vitamin deficiency in higher animals receiving vitamin analogues (pyrithiamine, glucoascorbic acid, isoriboflavin, β -acetylpyridine, etc.) and by encouraging results in the treatment of certain kinds of cancer with folic acid antagonists and with purine antagonists such as 6-mercapto-

purine, 2,6-diaminopurine, 6-chlorpurine, and pyrazolo purines. Enzyme inhibition is also the basis of the activity of a number of narcotics and of certain chemical warfare agents and insecticides such as tetraalkyl phosphates, dialkyl p-nitrophenylphosphates, and dialkyl fluorophosphates, which irreversibly inhibit esterases, including acetylcholinesterase.

Generally, enzyme inhibition may be of a number of kinds, viz., competitive inhibition of the kind typified by the malonatesuccinate competition for succinic dehydrogenase noted above, noncompetitive inhibition in which the inhibitor combines with the enzyme at a site other than the active site, and, less commonly, other kinds of inhibition in which, for example, the inhibitor combines with the enzyme-substrate complex, removes essential ions by chelation, and so on. Competitive inhibition may be distinguished by plotting the degree of inhibition against substrate concentration; in the competitive type of inhibition this is a linear function of substrate concentration, i.e., inhibition is inversely related to the ratio of substrate concentration to inhibitor concentration, while in the noncompetitive types in which the inhibitor does not combine with the enzyme at the active site, the affinity of the enzyme for substrate is not altered, and the rate of the enzyme-catalyzed reaction is not related to the ratio of the concentration of substrate to that of inhibitor. Such enzyme inhibitions may be reversible, or partially or completely irreversible.

The folic acid system. Inhibition among microorganisms may be illustrated by elaboration of the sulfonamide-p-aminobenzoic acid relationship. As described elsewhere (Chap. Five), p-aminobenzoic acid is an intermediate in the synthesis of folic acid, and folic acid is, in turn, a precursor of the formyl-folic acid (folinic acid) coenzyme. The synthesis of folic acid from p-aminobenzoic acid, glutamic acid, and the pteridine nucleus is antagonized by sulfonamides, and the inhibition is competitive. Sulfonamides do not, however, inhibit the conversion of folic acid to folinic acid; rather, this reaction is inhibited noncompetitively by 4-amino derivatives of folic acid, such as aminopterin and α -methopterin. The latter inhibition, then, prevents folic acid from acting as a formylating agent in 1carbon transfer reactions involved in the synthesis of at least some amino acids and nucleic acids.

Differences in the effect of sulfonamides on different kinds of microorganisms may be resolved in terms of their synthetic abilities, and it has been suggested that three general groups may be distinguished. The first is the gram-negative bacteria, which do not require preformed folic acid, and are inhibited by sulfonamides, and the inhibitory effect is not reversed by added folic acid. The second includes the gram-positive bacteria that do not require preformed folic acid, are inhibited by sulfonamides and the inhibition is reversed by folic acid. The third group, also gram-positive bacteria, is made up of those requiring preformed folic acid for growth and not sensitive to sulfonamides.

Limiting systems. In some instances analogues may compete with metabolites acting as coenzymes, but in general the evidence indicates that the competition occurs when the metabolite acts as a substrate rather than as a coenzyme. As a substrate, the metabolite is synthesized into a more complex molecule which functions as a coenzyme, or it may be degraded prior to synthesis. Further, various apoenzymes may compete, for folic acid for example, and the effective ratio of inhibitor to metabolite depends upon which enzyme system is involved. The requirements of different systems vary, and one of them is the limiting factor.

The sulfonamide sensitivities of bacteria which do not require p-aminobenzoic acid or folic acid for growth may be abolished by including the amino acids, purines, and pyrimidines in the culture medium which are synthesized in the reactions catalyzed by folic acid derivatives. When the necessity for the functioning of the limiting system is removed by supplying its end product, the concentration of antimetabolite is no longer inhibitory. If, however, the antimetabolite concentration is increased, inhibition is again apparent, another enzyme system has become the limiting factor, and again the inhibitory effect may be removed by supplying the end product of that system. Thus, the end products of the several systems involved which produce sparing effects can be arranged in an order. In the case of the colon bacillus, for example, methionine, purines, serine, and finally thymine or thymidine constitute such a series of which each member is dependent upon those preceding it. In this way studies of the nature of substances antagonizing the activity of the inhibitor may shed considerable light on the nature of the relevant reactions of synthesis.

Other inhibitors. There are a number of other instances of inhibition by analogues of essential metabolites of bacteria, produced by replacement of a carbonyl group with a sulfonic acid or sulfonamide radical or by a ketone, by substitution in the ring system of aromatic compounds, the replacement of alkyl side chains of ring systems with halogens, etc. Such are the sulfonic acid and sulfonamide analogues of nicotinic acid, pantolyltaurine (thiopanic acid), and pyrithiamine, the pyridine analogue of thiamine.

Effective chemotherapy of virus infections would appear to fall within this general concept, in the sense of selective inhibition of viral synthesis in the infected cell. $^{5, \, 51, \, 105}$ The prophylactic activity of N-methylisatin β -thiosemicarbazone noted above presumably has such a basis. Other compounds having activity have been studied also, notably vitamin B_{12} , guanine, and thymine analogues.

The inhibition of many picornaviruses in cell culture by 2-(hydroxybenzyl) benzimidazole and by guanidine¹⁰⁵ appears to be relatively specific, for these compounds are quite nontoxic for the host cells, and viruses of other groups, including poxviruses, herpesviruses, myxoviruses, reoviruses, and arboviruses, are not affected. The nature of the antiviral activity is not well understood, but there is reason to suppose that these agents function by inhibiting the production of virus-induced RNA polymerase.

It may be noted in passing that various other substances inhibit cellular RNA biosynthesis, e.g., actinomycin D and 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole, the latter by inhibiting the incorporation of adenosine into cellular RNA. Such inhibitors, particularly the former, have been useful in the elucidation of RNA function in biosynthetic reactions.

Halogenated deoxyuridines have been found to inhibit the replication of the DNA viruses of herpes simplex, vaccinia, and varicella⁷⁷ in cell culture and have very considerable utility in the chemotherapy of herpes keratitis in man. The halogen substitution is in the 5-position. The fluorine

compound has been found to inhibit the synthesis of thymine monophosphate and is not incorporated into the DNA. The iodine and bromine derivatives inhibit the utilization of thymine compounds, but not their synthesis, and are incorporated in DNA. Of these, 5-iodo-2'-deoxyuridine (IDU) has been used for therapeutic purposes in man and has been found to be effective in the treatment of experimental vaccinial keratitis.⁵²

Other antiviral agents function by inhibiting the initial absorption and/or penetration of the virus particle and are essentially prophylactic rather than therapeutic. Receptor-destroying enzyme (RDE) is effective in the prevention of influenza virus replication by depolymerization of the receptor substrate on the surface of the host cell, and some mucopolysaccharides of microbial origin presumably function by offering an alternative to the host cell receptors. Considerable interest has attached to the antiviral activity of amantadine (1adamantanamine HCl) for the influenza virus; its activity appears to be prophylactic rather than therapeutic in experimental infections in animals30 and man,91 but the nature of the inhibitory effect is not known.

Inhibition may also be indirect or nonspecific in the sense that compounds other than structurally related analogues may be inhibitory. In the first instance the substance applied as an inhibitor may be inactive but metabolized to an active form. The wellknown inhibitory effect of fluoro-acetate is apparently due to its metabolic conversion to fluorocitric acid, which is an inhibitor of aconitase. There is evidence also that exchange reactions with the coenzyme may occur with some of the pyridine compounds; isonicotinic acid hydrazide has been found to undergo exchange with nicotinamide, and the corresponding NAD analogues have been isolated. Inhibition by structurally unrelated compounds occurs also as in the inhibition of invertase by certain basic dyes: sucrose and other sugars compete with the dye for the enzyme, suggesting that a variety of chemically different substances having appropriately distributed charges may compete for acidic or basic groups on the enzyme to produce inhibition of its activity.

The nature of the antibacterial activity of enzyme inhibitors cannot be sharply differentiated from the antibacterial effects of substances having utility as disinfectants. While the lethal action of disinfectant substances is a net effect, as pointed out earlier, probably the most important single effect, though not necessarily always the quantitatively predominant one, is inactivation of enzyme systems. Thus, the affinity of heavy metals for sulfhydryl groups, including those of enzymes, is well known, and the bisphenols, such as hexachlorophene, strongly inhibit succinoxidase, cytochrome oxidase, and lactic dehydrogenase; still other examples of this kind of effect of disinfectants have been noted above.

ANTIBIOTIC SUBSTANCES^{23, 28, 115}

The mutual relationships occurring among the microorganisms are analogous to those well known among other living organisms, ranging from synergistic and beneficial associations to antagonistic relationships. Synergism, the relation in which a group of two or more kinds of microorganism has capabilities greater than the sum of those of the members of the group considered separately, is apparent in many situations, as different as the cyclic transformations of organic matter in the soil, and the dual or multiple etiology of infectious diseases such as swine influenza or gaseous gangrene.

The antagonistic relationship is often apparent between saprophytic and parasitic pathogenic microorganisms. In a general way, most pathogenic microorganisms are closely adapted parasites, poorly equipped to survive the rigors of intense competition with mixed microbial flora outside the body of the host. This appears to be the basis of the ability of the typhoid bacillus, for example, to survive for two to three weeks in sterile water, but only a few days in natural waters and even less in polluted waters. Similarly, the pathogenic microorganisms present in the bodies of persons dead of infectious disease, with the exception of the relatively rare spore-forming pathogens such as the anthrax bacillus, are unable to survive in the soil, and there is no hygienic objection to earth burial of such bodies.

Thus the microbial population, both animal and plant, of natural habitats such as soil and water, is determined by the

mutual relationships among the members of the population as well as other environmental conditions. The pathogen appears as a newcomer, too poorly adapted to establish an ecological niche in such habitats, and is unable to survive.

The antagonistic relationship is often attributable to the elaboration of substances by one kind of microorganism which are toxic or inhibitory to other kinds. The bluegreen pigment pyocyanin, a phenazine comproduced bv Pseudomonas aeruginosa (Ps. pyocyaneus), is actively toxic for very many kinds of bacteria and was, in fact, known as early as 1860 as the chloroform-soluble substance giving color to the "blue pus" of pyogenic infections with this microorganism-and before bacteria were known to be etiologically related to disease.

Such antimicrobial substances, originating as products of metabolism of living organisms, are known as antibiotics. 14 Such substances, elaborated under natural conditions in environments such as soil, which contains enormous numbers of a wide variety of microorganisms, play a significant role in microbial ecology. 107 In retrospect, saprophytic microorganisms present in soil seem a self-evident possible source of antimicrobial substances affecting pathogenic bacteria with some specificity, but the first antibiotic applicable to the treatment of infectious disease, penicillin, was not discovered until 1929 by Fleming. Since different kinds of bacteria differed in their sensitivity to its growth-inhibiting activity, penicillin was regarded as useful only in the preparation of selective mediums for the primary isolation of bacteria resistant to it such as Hemophilus species. It was not until 1940 that the concept of selectivity of action was extended to include man, with the consequence that its lack of toxicity for man in concentrations effectively inhibiting the growth of pathogenic microorganisms in vivo led to its use as a chemotherapeutic agent.

It is a common experience of those examining large numbers of isolation plates to find colonies of microorganisms surrounded by zones of no growth of other kinds of microorganisms present in the culture. It is but a single step from such observations to the inoculation of nutrient medium in a petri dish with a test bacterium so that a uniform film or growth will develop,

followed by a second inoculation with a rich source of varied microbial flora, commonly soil, by the streak or other dilution method to give isolated colonies. After incubation the culture is examined for halo-like zones of growth inhibition of the test organism about colonies growing up from the second inoculation. When such zones are observed. the appropriate colony is subcultured and the subculture tested for the presence of soluble growth-inhibiting activity for the test bacterium. The activity may be purified by various appropriate chemical methods and tested for both antimicrobial activity and toxicity to an experimental animal. If the toxicity is sufficiently low in relation to antimicrobial activity, it is tested for chemotherapeutic activity in experimental infections.

This procedure is relatively simple and so has been widely applied with the demonstration of many hundreds of kinds of antibiotic activity. Fungi, actinomycetes and molds, have been the most prolific source of antibiotics, although some are of bacterial origin. Unfortunately, the great majority of these are toxic, some highly toxic, to animals and are discarded from consideration as possible chemotherapeutic agents. Nevertheless, some 340 useful and potentially useful antibiotics are described in one compilation,88 of which some 20-odd are in active use. All show a greater or lesser degree of specificity of action on different kinds of bacteria, often associated with the Gram staining reaction, and the range of kinds of bacteria sensitive to the action of an antibiotic is spoken of as its "spectrum" of activity. Of the many antibiotic substances, only the most common will be considered here.

Antibiotic substances from bacteria. A variety of antibiotics are formed by the aerobic sporulating bacteria of the genus Bacillus. The first of these to be studied as a chemotherapeutic agent was isolated by Dubos in 1939 from *B. brevis*, and found to be an alcohol-soluble, water-insoluble polypeptide which was named tyrothricin.

Further investigation made possible its separation into two components, gramicidin and tyrocidin, of somewhat different properties though both contain "unnatural" amino acids of the d series. Gramicidin is a large cyclopeptide containing relatively large amounts of tryptophan and is effective only on gram-positive bacteria. A

closely similar substance, gramicidin S, is formed by a thermophilic variety of B. brevis which differs in that it has considerable activity against gram-negative bacteria and is a cyclopeptide hydrochloride with one free amino group, no free carboxyl, and one hydrochloride residue, made up of one residue each of *l*-ornithine, *l*-proline, *l*-valine, l-leucine, and d-phenylalanine. Tyrocidin is active against both gram-positive and gram-negative bacteria in vitro, but its activity is almost completely inhibited by serum proteins. These antibiotics are surface-active substances, and their antibacterial activity is perhaps attributable to their destructive effect on the cell wall of the bacterium.

A number of antibiotic substances have been isolated from strains of B. subtilis which vary somewhat in their properties and antibiotic activity, though all appear to be polypeptide in nature. Subtilin is effective chiefly on gram-positive bacteria and certain of the acid-fast bacilli. It has low toxicity and is effective in the therapy of experimental infection of mice with pneumococci and guinea pigs with anthrax. Bacitracin is similar to subtilin and effective on gram-positive bacteria, but its utility is limited by its nephrotoxicity. Bacillin is highly active on both gram-positive and gram-negative bacteria in vitro and is only moderately toxic, but, like tyrocidin, its activity is destroyed by blood and it is completely ineffective as a chemotherapeutic agent. Eumycin does not affect gram-negative bacteria but has in vitro activity against the diphtheria and tubercle bacilli and some of the fungi. Its toxicity is low but its therapeutic efficiency is not known. Licheniformin resembles subtilin in its properties and is regarded by some workers as identical with

Other antibiotics produced by bacteria are designated collectively as bacteriocins, 48,69 and more specifically as colicins, megacins, etc. Some of the bacterial pigments show antibiotic activity, including violacein from Chromobacter violaceum, phthiocol formed by the tubercle bacillus, and iodinin from Chromobacter iodinum.

Antibiotics produced by higher fungi.¹⁰ So far the most prolific source of chemotherapeutic antibiotics has been the higher fungi, that is, certain molds but especially the actinomycetes of the genus Streptomyces. These substances are relatively

simple compounds of molecular weights of less than 1000. Some of those which are toxic to animals and have no chemotherapeutic potentiality have been useful in other ways. Actinomycin A, for example, is a relatively specific inhibitor of RNA synthesis because it blocks the action of DNA-dependent RNA polymerase. Since this agent complexes with guanine in double-stranded DNA, RNA-primed syntheses are not inhibited, thus allowing the separation of viral RNA synthesis from that of the host cell.

Penicillin. 13, 95 Penicillin is not a single substance, but a group of closely related compounds differing from the parent β lactam monobasic carboxylic acid with respect to the side chain. These substances are formed by Penicillium notatum, 22 other species of Penicillium, and seven species of Aspergillus. The six common naturally occurring forms are shown in the accompanying table. Two other penicillins are produced by biosynthesis. One is cephalosporin N, produced by a species of Cephalosporium, which has been shown to be (D-4-amino-4-carboxy-n-butyl) penicillin. The other is penicillin V, phenoxymethyl penicillin, which is formed by P. chrysogenum when N-2-hydroxyethyl phenoxyacetamide is supplied as a precursor, which, in contrast to other penicillins, is stable as the free acid.

In addition, various relatively insoluble, and therefore less readily excreted, forms of penicillin have been prepared, including benethamine (N-benzyl- β -phenylethyl penicillin), hydrabamine (N,N' dihydrobutyl ethylenediamine dibenzyl penicillin) and benzathine (Benzethacil, Bicillin) (N,N'

dibenzyl diethylenediamine penicillin G.). Of these various forms of penicillin, penicillin G (phenacetyl penicillin) is the one generally available as a stable salt because it is most readily produced on a large scale.

The alteration of penicillins produced by fermentation to give "synthetic" penicillins has resulted in the preparation of a number of compounds having valuable properties. The phenoxy compounds include α -phenoxymethyl, α -phenoxyethyl, and α -phenoxypropyl penicillins, 109 which are acidresistant and can be given by mouth. A compound prepared from naturally formed 6-aminopenicillanic acid, $6[D(-)\alpha$ -aminophenylacetamido] penicillanic acid (Penbritin), is also acid-resistant and, in addition, has a considerably broader spectrum of activity than the other penicillins.^{79, 96} Special interest has attached to sodium 6-(2,6-dimethoxybenzamido) penicillinate (Celbenin, methicillin, Staphcillin), because the alteration in its structure is sufficient that it is not destroyed by penicillinase and is, therefore, effective against penicillinresistant staphylococci.61

Penicillin is produced by biosynthesis,¹⁷ although it has been synthesized, not because of the complexity of the molecule, but because it is relatively unstable. The synthetic product is not yet competitive with that produced by fermentation.

Naturally Occurring Penicillins

NAME	SYNONYM	SOURCE	R
Penicillin F Dihydro F penicillin	Penicillin I Gigantic acid	P. notatum P. chrysogenum A. giganteus	$CH_3CH_2CH = CHCH_2$ $CH_3CH_2CH_2CH_2CH_2$
Flavicin Flavicidin Penicillin G	F type Penicillin II	A. flavus P. notatum P. chrysogenum	$CH_3CH = CHCH_2CH_2$ CH_2
Penicillin X	Penicillin III	P. notatum P. chrysogenum	$HO \longrightarrow CH_2^-$
Penicillin K	Penicillin IV	P. notatum P. chrysogenum	CH ₃ CH ₂ CH ₂ CH ₂ CH ₂ CH ₂ CH ₂

Penicillin is most active on gram-positive bacteria, but some gram-negative forms such as the gonococcus and meningococcus are susceptible to it, as are most spirochetes. It appears to act primarily as a bacteriostatic agent though high concentrations may be bactericidal. In general it is more active than the sulfonamides and less so in vitro than the triphenylmethane dyes.

Streptomycin. This antibiotic is formed by Streptomyces griseus and was isolated by Waksman in 1943. It is a basic glucoside and may be changed to dihydrostreptomycin by hydrogenation. The structural formula of dihydrostreptomycin is shown in 7, the starred carbon indicating the point at which hydrogen is added; in streptomycin this alcohol group is an aldehyde. These substances differ from penicillin in their activity in that they are effective on gram-negative bacteria and also on the tubercle bacillus. The susceptibility of bacteria to streptomycin is variable from strain to strain, and the sensitivity of the infecting organism, particularly in the case of green streptococci, should be tested. A serious drawback to the use of this antibiotic is the facility with which sensitive bacteria become resistant to it.

Streptomycin and dihydrostreptomycin do not differ in antibacterial activity from one another except that some strains of Salmonella are less sensitive to the dihydro compound.

Chloromycetin). This antibiotic, formed by Streptomyces venezuelae, was described in 1947 by Ehrlich and his associates. Its structure has been determined, and it is now prepared by synthesis. the only antibiotic so prepared on a large scale. The structure is of some interest because aromatic nitro compounds and derivatives of dichloracetic acid are relatively rare in nature (8). This antibiotic. together with the tetracyclines, makes up the group of "broad-spectrum antibiotics," i.e., those that are effective against a wider variety of microorganisms than either penicillin or streptomycin. Chloramphenicol is effective not only against the gram-positive bacteria, but also the gram-negative bacteria and the typhoid bacillus in particular. It is also active against the rickettsiae and the microorganisms of the psittacosis-

8.

lymphogranuloma group. Its efficacy in typhoid fever is unique, and it is the drug of choice in the treatment of this disease. It is, however, relatively ineffective in bacillary dysentery.

Chloramphenicol is relatively nontoxic by mouth and is effective by the oral route, for it is readily and almost completely absorbed from the gastrointestinal tract, and only occasionally nausea, vomiting, or diarrhea is produced. There have, however, been a number of reports of aplastic anemia following the administration of this antibiotic, perhaps to be expected since it is a nitro compound.

Tetracyclines. Chlortetracycline (Aureomycin), produced by Streptomyces aureofaciens, and oxytetracycline (Terramycin), produced by Streptomyces rimosus, were found independently, the former in 1948 by Duggar and his associates and the latter by Finlay and his associates in 1950. It soon became apparent that the two resembled one another closely, and it was subsequently shown that they are isomorphic tetracycline compounds, chlortetracycline containing nonionic chlorine at the sixteenth carbon and oxytetracycline a hydroxy group on the twelfth carbon. The parent compound, tetracycline (Achromycin, Polycycline,

Chlortetracycline has a Cl attached at *, while oxytetracycline has a hydroxyl group attached at **.

Tetracyn, Stechlin, Panmycin), is obtainable by catalytic reduction of chlortetracycline and also by fermentation by a species of Streptomyces.

Chlortetracycline is distinguished by its golden yellow color. The chloride is stable, but in neutral solution the antibiotic decomposes relatively rapidly at incubator temperatures. This makes for some difficulty in the microbiological assay of its activity, and cultures need to be observed after short periods of incubation. Its bacterial spectrum is closely similar to that of chloramphenicol, but it is not as effective in the treatment of typhoid fever.

Chlortetracycline is relatively nontoxic on oral administration; untoward reactions are mild and include nausea, vomiting, and diarrhea. Parenteral inoculation is painful and results in an erythematous tender area at the site of intramuscular inoculation. In practice the requirement for parenteral administration is limited, since the drug is well absorbed from the gastrointestinal tract.

Demethylchlortetracycline, having one less methyl group, reaches blood levels more slowly, but renal clearance is low and blood levels tend to persist. It is reported to have equal, if not greater, antibacterial activity than the parent compound.21

Oxytetracycline is a white substance which in neutral solution is more stable than chlortetracycline but resembles it closely in antibacterial activity, efficacy when given by mouth, and other factors. In inhibitory concentrations it blocks the utilization of ribonucleic acid reserves that are lost to the surrounding medium after depolymerization to mononucleotides. It also inhibits respiration in the presence of 4-carbon dicarboxylic acids, pyruvate, tricarboxylic acids, and α -ketoglutarate.

The parent compound, tetracycline, does not differ significantly from the chlor- and oxy-derivatives in antibacterial activity.

Chemical classification. 1, 67 While antibiotics are chemically diverse, most of them fall into three general groups, viz., (1) those derivable from amino acids, including penicillin, chloramphenicol, bacitracin, polymyxin, and cycloserine; (2) those derivable from acetate, including the tetracyclines, griseofulvin, the erythromycin group of macrolides, and the conjugated polyenes, amphotericin B and nystatin; and (3) those derivable from sugars, including streptomycin, kanamycin, and neomycin.

The macrolide antibiotics are characterized by a lactone ring, a ketone function commonly an α - β unsaturated system, and a deoxyamino sugar containing a dimethyl amino group. Some of these are useful as esters formed by attachment of acyl groups to various hydroxyl groups. Thus the propionyl ester (as the lauryl sulfate salt) and the ethyl carbonate ester, esterified in the hydroxyl group of desosamine, of erythromycin have practical utility.

The polyene antibiotics, having the general structure (CH=CH)_n, also contain large lactone rings, but are set apart from the macrolides both chemically and biologically. They are active against a variety of fungi, including both yeasts and filamentous forms, but are inactive against bacteria or actinomycetes. Of these, amphotericin B, a heptaene polyene, and nystatin, a dienetetraene polyene, have been most useful in the treatment of a variety of fungous diseases (Chap. Thirty-two).

Mechanisms of action.^{65, 66} The marked activity and specificity of the antibiotics has stimulated great interest in the nature of the antimicrobial effects they produce. Sufficient information is now at hand to allow a tentative grouping of these substances with respect to the mechanisms of

their action.

Cell wall synthesis. The penicillins, and a number of other antibiotics including cycloserine, bacitracin, novobiocin, vancomycin, and ristocetin, appear to affect primarily the cell walls of sensitive bacteria. It will be recalled (Chap. Three) that bacterial cell walls are made up of a number of substances which are structurally unique in that they are not found in mammalian cells, e.g., the "unnatural" isomers of glutamic acid and alanine, α - ϵ -diamino pimelic acid, muramic acid, and the teichoic acids. The chemical uniqueness of the cell wall structure of bacteria may contribute in large part to the lack of toxicity of the penicillins, e.g., to the mammalian host.

The susceptibility of the actively growing bacterial cell to penicillin and its relative resistance in the resting stage suggest that an active synthetic process is affected. Further, in the presence of penicillin, actively multiplying cells tend to continue to grow for a time, but do not divide, and may eventually be stripped of cell wall with the formation of protoplasts. Concurrently, uridine diphosphate derivatives of muramic acid and muramic acid peptides accumulate, and the accumulation correlates with the relative antibacterial activity of the several penicillins. On the basis of such observations and other more detailed evidence of the same general nature, it seems clear that a primary effect of penicillin on sensitive bacteria is that of inhibition of cell wall mucopeptide synthesis.

Available evidence indicates that, although the same general consequence is produced by cycloserine, the mechanism differs. Growth inhibition by this antibiotic is reversed by p-alanine. The synthesis of

muramyl peptide by the growing bacterium involves the addition, one by one, of single amino acids to uridine diphosphate-N-acetyl-glucosamine-3-O-lactic ether to give a tripeptide terminating in lysine. The terminal dipeptide, D-alanyl-D-alanine, is then added as a unit. In the presence of cycloserine, there is an accumulation of the muramyl tripeptide. The antibiotic does not prevent the addition of D-alanyl-D-alanine, but competitively inhibits alanine racemase and D-alanyl-D-alanine synthetase. There is not yet sufficient information about the mechanisms of action of the other antibiotics which appear to affect cell wall synthesis.

Permeability. The cyclic peptide antibiotics—the polymyxins, tyrocidin, and gramicidin S—and the polyene antibiotics produce an immediate and rapid leakage of low molecular weight substances, e.g., constituents of the amino acid pool, which is independent of growth of the bacteria in the presence of the antibiotic. The precise mechanisms are as yet quite uncertain and probably are different. Thus the polyenes are antagonized by sterols, and sterols are constituents of fungal cell membranes but not of bacteria, which are resistant to these antibiotics.

Protein synthesis. The antibiotics which inhibit net synthesis of protein in affected bacteria include chloramphenicol, streptomycin (and the similar antibiotics streptothricin, kanamycin, neomycin, viomycin, and paromomycin), the tetracyclines, and puromycin. The last is sufficiently toxic that it has little chemotherapeutic value. The biosynthesis of protein is considered elsewhere (Chap. Five), but it may be summarized here as follows: Amino acids are activated by ATP as aminoacyl adenylates, which in turn react with sRNA. The aminoacyl-sRNA complexes are transferred to determined positions on the mRNA template in association with ribosomes (Chap. Three), and the polypeptide chain is built up by linkage through peptide bonds. The antibiotic activity associated with inhibition of protein synthesis may be considered against this background.

Chloramphenicol does not inhibit the synthesis of DNA and RNA; in fact these syntheses continue in concentrations of the antibiotic which stop protein synthesis, and RNA synthesis may even be stimulated. Studies on cell-free systems have indicated that this antibiotic does not affect the activa-

tion of amino acids, their transfer to sRNA. or the binding of sRNA to ribosomes, but that it does block the transfer of amino acids from xRNA to ribosomes. It combines with ribosomes to produce a complex having a sedimentation constant of 15 S which behaves as loosely coiled polyelectrolyte, like RNA rather than ribosomes, but the nature of the complex remains uncertain. It is also uncertain that protein synthesis is inhibited by preventing the combination of mRNA with the ribosome. It seems to be clearly indicated, though, that inhibition of protein synthesis by this antibiotic is intimately associated with its effects on ribosomes.

Streptomycin affects sensitive bacteria in a variety of ways, including inhibition of the oxidation of certain substrates, breakdown of RNA, and damage to cell membranes, as well as inhibition of protein synthesis. It seems probable that the first two effects are secondary to effects on permeability and protein synthesis, but which of these is secondary to the other is obscure. Studies on protein synthesis have shown that streptomycin is bound to ribosomes, and that the mRNA binding site on the 30 S component is altered so that the information carried by mRNA is misread, with synthesis of abnormal nonfunctional protein. But it is not at all clear how this effect may be related to changes in permeability, reflected as a rapid loss of potassium.

Tetracyclines inhibit adaptive enzyme formation (i.e., protein synthesis) and phosphorylation but not respiration of mitochondria, and the blocking of phosphorylation of adenine nucleotides suggests the possibility of interference with the Krebs cycle. They also function as chelating agents, and their inhibitory effects may be reversed by metal ions. They have been found to inhibit the transfer of amino acids from aminoacylsRNA to ribosome protein, but not the binding of sRNA to ribosomes: such data have been considered to suggest that these antibiotics inhibit the formation of peptide

bonds.

THE APPLICATION OF CHEMOTHERAPEUTIC AGENTS

Since the antimicrobial activity of the chemotherapeutic agents (not only the antibiotics but also sulfonamides and the synthetic compounds such as p-aminosalicylic acid and thiosemicarbazones) is primarily growth-inhibitory, it is assayed by growthinhibition tests. Conversely, the relative sensitivity of kinds and strains of microorganisms is determined by test against standard concentrations of such substances.

Assay of activity. The assay methods are of two general kinds, the tube method and the disc method. In the former, serial dilutions of the substance are prepared in a standard liquid culture medium, and the culture tubes (or flasks) are inoculated with a constant number of bacteria, incubated, and examined for growth. Thus the activity may be measured as the smallest amount of the active substance that prevents growth under the specified conditions. When large numbers of tests are to be carried out, as in the routine testing of bacterial sensitivity. the method is relatively time-consuming. and so is not often used.

The so-called disc method is based on the zone of growth inhibition surrounding a colony of antibiotic-producing microorganisms growing in culture in which the test microorganism is growing as a continuous film on the surface of the agar medium as described earlier. The first such method devised consisted in placing small, sterile, glazed ceramic or glass cylinders vertically on the surface of the uniformly inoculated agar medium and pipetting into them serial dilutions of the substance under test. The activity diffuses outward in the culture medium and, within limits, the activity of the preparation may be determined by comparison with the zone of growth inhibition produced by the standard. Six such cylinders may be appropriately arranged in the usual petri dish culture without overlap of inhibition zones. The standard on which such an assay is based is arbitrarily determined. The activity of penicillin, for example, was originally defined as the Oxford unit, a standard strain of Staphylococcus being used as the test microorganism. This was subsequently replaced by the International Unit, which approximates the Oxford unit; the standard preparation is one of crystalline sodium penicillin G, containing 98.5 per cent of penicillin G. It contains 1670 IU per ml., and the IU is 0.5988 ± 0.5 per cent µg.45 Similarly the IU of streptomycin is arbitrarily defined, but almost equivalent in activity to that of 1 μ g of pure dihydrostreptomycin base,41 and the oxytetracycline standard preparation contains 900 IU per mg.⁴² Similar international standards have been defined for tetracycline⁴³ and for erythromycin.⁴⁴

This kind of assay is not confined to the measurement of the activity of chemotherapeutic substances but has also been applied to other antimicrobial substances such as the quaternary ammonium compounds. In contrast to assays of the phenol coefficient type, it does not depend upon a sterility end point and measures growth-inhibitory rather than lethal activity.

Sensitivity tests. The great bulk of such assays, however, is directed toward the measurement of the sensitivity of microorganisms isolated from disease conditions as a guide to effective chemotherapy. While certain generalizations may be made, viz., gram-positive bacteria are sensitive to penicillin and gram-negative bacteria, with certain exceptions, are not, and the converse relation holds true with streptomycin, too much reliance cannot be placed upon them for two reasons. First, bacteria may become resistant to chemotherapeutic substances (Chap. Seven) so that the sensitivity of a given strain may not be predicted with complete confidence. Second, some kinds of bacteria, such as α -hemolytic streptococci, vary widely from strain to strain in their sensitivity to antibacterial chemotherapeutic agents. Testing for sensitivity of isolates has, then, become a routine diagnostic adjunct.

The sensitivity spectrum so determined depends upon the context from which it is taken. The usual spectrum of a given chemotherapeutic substance is commonly derived from stock cultures of bacteria, but that found with isolates from infectious processes may differ, usually in that resistant strains of bacteria "normally" sensitive to a given agent may be found. In a general way, such a shift is most pronounced in isolates from populations of hospitalized persons, and tends to be related to the kinds of chemotherapeutic agents used and the extent to which they are used. The utility of a given chemotherapeutic substance thus depends upon a number of factors, and the spectrum of its activity is more realistically given by the proportions of bacterial strains found to be sufficiently sensitive to it so that it has utility as a therapeutic agent. An illustration of such a practical spectrum of activity is given in the accompanying table.

The diagnostic sensitivity test. The sensitivity test applied for this purpose is considerably simplified by the use of filter paper discs impregnated with appropriate concentrations of the chemotherapeutic substances. This kind of test is recommended as an international standard,113 and for routine tests with pathogenic bacteria (excluding tubercle bacilli) the following antibiotics should be used: erythromycin, novobiocin, vancomycin, bacitracin, penicillin, tetracycline, chloramphenicol, neomycin, streptomycin, and polymyxin. The discs are placed upon the surface of the agar culture medium uniformly inoculated with the test bacterium. There are many variations on this scheme, including cogwheel-shaped pieces of filter paper having the projections impregnated with different substances, circular pieces with impregnated projections toward the center, etc. In any case, two concentrations of the antibacterial substances are usually used, a "high" and a "low" concentration. For example, the concentrations of penicillin are 10 and 1.5 units, of tetracycline 25 and 4 units, erythromycin 10 and 1 unit, streptomycin 100 and 10 μ g, etc. The "high" concentration is intended to approximate the maximum blood levels of the substance attainable, but this is not always true. For instance, for technical reasons, sensitivity to streptomycin for therapeutic purposes is best shown by the indicated concentrations, although in this case the blood level attained is more nearly 10 μ g than 100 μ g because of toxicity.

After incubation the test is read as the diameter in millimeters of the zones of growth inhibition, but judgment must be used since the size of the zone of inhibition is also a function of the rate of diffusion of the activity into the culture medium. A high degree of sensitivity, for instance, is indicated by a maximal zone of inhibition around the "low" concentration even though that around the "high" concentration may not be larger because of the diffusion factor. This method of assay of bacterial sensitivity is widely applied and gives remarkably consistent results in view of its relative crudity.

Secondary infections. 63 The microbial flora of man and other animals is diverse, and when an antimicrobial agent is administered for therapeutic purposes, there is an inevitable side effect on the normal flora due to the selective toxicity of the agent.

Percentage of Representative Microorganisms Isolated from Infectious Processes Sensitive to Antibiotics*

Microorganism							Antibiotics	tics					
KIND	NUMBER OF STRAINS	AMPI- CILLIŅ	СЕРНА- LOTHIN	CHLORAM- PHENICOL	COLIS-	ERYTHRO- MYCIN	FURA- DANTIN	KANA- MYCIN	NOVO- BIOCIN	PENI- CILLIN	STREP- TOMYCIN	SULFON- AMIDES	TETRA- CYCLINE
Staphylococci St. aureus St. albus	472 37	34 62	99	94 81	3	81 88	81 94	90 90	93 100	41	91 14	62 49	45 54
Streptococci Str. fecalis	719	47	37	81	_	77	99	10	41	76	_	0	11
Beta hem. strep. Non-hem. strep.	144	80	95 90	91	11 8	86	84 84	23 38	65 65	88	32,3	10 29	48 71
Alpha strep.	69 =	87	90 00	91 94		86 100	78	50 50	100	91	21	30 83	. 52 89
Hemophilus influenzae	10	06	20	100	100	100	100	100	06	100	80	80	08
Neisseria gonorrhea	10	80	100	100	0	100	100	0	100	100	09	100	100
Escherichia coli	1,462	49	2.9	88	94	0.4	84	711	0.7	0	14	711	4
Klebsiella-Aerobacter	729	6	20	82	91	0	38	65	0	0	12	<i>L</i> 9	52
Citrobacter	63	П	23	37	82	0	76	98	0	0	3	24	0
Proteus mirabilis	370	68	78	87	w	00	20,0	88	26	11	43	84	000
Proteus rettgeri Proteus morgani Proteus vulgaris	22 6 12 .	33 × 0	23 16	58 28 58	746	000	000	80 80 80	10 25	0 & 0	4 4 4 4 4 4 4 4 4 4 1 4 4 1 4 4 1 4 4 1 4 4 1	35	13 0
Pseudomonas aeruginosa Pseudomonas species	309	-8	3	15	79	0	3.6	32	60 KO	0 %	6 7	65	2
The state of the s	-												

*Organisms are from the first 400 tested each month from July 1966 through June 1967 in the Clinical Microbiology Laboratory of the University of Chicago Hospitals and Clinics. The percentages shown here are of organisms considered sensitive or moderately sensitive, with zones of inhibition of 18 mm. or more in

diameter (11 mm. for cephalothin) with the high concentrations in the disc test. These data are not necessarily characteristic of nonhospitalized infections, those of other hospitals, or of this hospital at other times. (Compiled by Dr. R. S. Benham and Miss Isabelle Havens.)

In the normal individual the character of the microbial flora is determined by many factors, and of these a significant element is the interrelationships of the microorganisms present in, for example, the large bowel. In this case, in addition to the quantitatively predominant coliforms, other gramnegative bacilli such as Proteus, sporulating anaerobes such as Cl. welchii, α-hemolytic streptococci or enterococci, staphylococci, fungi such as yeasts, and filamentous forms such as Candida albicans are present in small proportion. When an antimicrobial substance is introduced, this flora changes in character with the emergence of minority forms that, when present in greatly increased proportions, assume some degree of pathogenicity. This effect is most commonly observed following oral administration of broad-spectrum antibiotics but may also occur when other antimicrobial substances and other routes of administration are used.

Thus secondary infections may occur which are quite unrelated to the disease condition for which therapy was initiated. There are three kinds of more common consequences. First moniliasis or candidiasis (Chap. Thirty-two) may develop by uninhibited, or possibly stimulated, proliferation of the fungus C. albicans with consequences ranging from, for instance, local irritation in the bowel, anus, or vagina, to a generalized, sometimes fatal, infection, especially in young children. Second, with selective inhibition of other components of the intestinal flora, staphylococci, often resistant forms, may become predominant to produce diarrheal disease and occasionally fatal staphylococcal toxemia. Third, when the growth of coliforms and enterococci is markedly depressed, forms such as Proteus and Pseudomonas may grow freely and invade the tissues to produce infections of the kidneys or urinary bladder. Still other effects may be produced by intensive antibiotic therapy. For example, the bacterial flora of the mouth and throat often become predominantly coliform in character, with coliform throat cultures not uncommonly encountered in the diagnostic laboratory, and it is believed by some that some strains of coliform bacilli, especially hemolytic forms, may produce inflammatory changes in the throat.

The overall effect of the general use of chemotherapeutic agents is illustrated in

compiled data from the Boston City Hospital from 1935 to 1957.²² Staphylococcal infections of the blood and meninges increased greatly, and there has been a coincident enormous increase in bacteremia and meningitis due to gram-negative bacilli, viz., Proteus, Klebsiella (coliform), and Pseudomonas. The mortality from bacteremia decreased from 1935 to 1947, but thereafter rose rapidly until by 1957 it was 50 per cent higher than in 1935.

Combined therapy.⁶ In view of the selective action of antimicrobial substances, their administration in combination is inevitable, both to broaden the antimicrobial activity of the preparation in the absence of precise diagnosis of the etiological agent in treatment of mixed infections, and to minimize the secondary infections occurring during intensive therapy described above and to reduce the probability of the development of resistance by the microorganism (Chap. Seven).

Various combinations, sometimes mixtures and sometimes compounds, have been prepared. Among the mixtures, the combination of streptomycin or isoniazid with p-aminosalicylic acid is almost routinely used in the treatment of tuberculosis; this is something of a unique situation in that the disease requires prolonged therapy which favors the development of bacterial resistance, and because of the facility with which the bacilli become resistant to streptomycin and, to lesser extent, to isoniazid. Other combinations include the admixture of antifungal substances such as candicidin, fungicidin, or undecylenic acid with the broad-spectrum antibiotics, especially the tetracyclines, to minimize fungal overgrowth during therapy, and various combinations of antibiotics such as tetracycline plus nystatin (Mysteclin) or plus oleandomycin (Sigmamycin, Signemycin).

Compounds of antibacterial substances include: streptomycyclidene isonicotinyl hydrazide sulfate (Streptohydrazid); Streptobine, a stable salt containing 48 per cent dihydrostreptomycin, 34 per cent isoniazid, and 18 per cent pyruvic acid; Hiconstarch, a polymer prepared from periodate-oxidized potato starch by condensation with equimolar proportions of isoniazid and p-aminobenzalthiosemicarbazone; a compound of isoniazid and p-aminosalicylic acid (Pasiniazide, Dipasic); isonicotinyl hydrazone

benzaldehyde metasulfonic acid (G605, Sulfoniazide); etc. The value of such preparations is not established.

Synergism and antagonism. Contrary to what might be supposed, the effect of combination of two substances demonstrable with a single species of microorganism susceptible to both is not necessarily an additive one. This phenomenon has been studied by a number of workers, especially by Jawetz and his colleagues⁴⁹ and by Klein and his coworkers.⁶⁰ Klein has summarized the kinds of effect produced by drug combinations both in vitro and in vivo as follows:

- (1) A synergistic effect may be defined as that in which the activity of two drugs in combination is greater than that obtained by doubling the concentration of either alone.
- (2) An additive effect is that in which the antibacterial activity of the combination is greater than that of either drug alone, but less than that obtained by doubling the concentration of either.
- (3) The effect is one of interference when the antibacterial activity of the combined drugs is not greater than that of either drug alone.
- (4) Two drugs are said to be antagonistic when the antibacterial activity of the combination is less than that obtained with either drug alone.

Effects falling into one or the other of the above categories have been observed with mixtures of sulfonamides with antibiotics and with mixtures of different antibiotics but are sometimes difficult to interpret. For example, one of the most widely tested combinations is that of streptomycin with p-aminosalicylic acid in the therapy of tuberculosis; this combination appears to be invariably synergistic. The combination of penicillin and sulfonamides, however, is initially antagonistic, but this is transitory and the effect eventually becomes synergistic.

Jawetz has generalized the interrelationships among the antibiotics by placing them into two groups: the first is made up of penicillin, streptomycin, bacitracin, and neomycin; and the second of Aureomycin, chloramphenicol, and Terramycin. He has found that members of the first group are often synergistic with each other and never antagonistic, while pairs of the second group show only an additive effect. The consequence of combining antimicrobial substances of different groups is variable and dependent upon the sensitivity of the microorganism to the drug in the first group; if it is highly sensitive, the second antimicrobial substance will interfere, but if it is relatively resistant the combination may be synergistic. He has suggested the following possible explanations:

- (1) The two antimicrobial substances combine chemically to give a more active compound.
- (2) One antimicrobial substance is effective on those bacteria that are resistant to the other.
- (3) One antimicrobial substance affects actively multiplying bacteria and the other viable but nonreproducing microorganisms.
- (4) One antimicrobial substance potentiates the other indirectly, for example, by decreasing the permeability of the cell wall.
- (5) The combination of antimicrobial substances simultaneously blocks more than one metabolic pathway, preventing a facile shift by the microorganism in the presence of a single antimicrobial substance.

Of these, the first is quite unlikely on the basis of present evidence. The second is probably the explanation of the PAS-streptomycin synergism in the treatment of tuberculosis. The third is probably the explanation of the behavior of sulfonamidepenicillin combinations; *i.e.*, the sulfonamides are primarily bacteriostatic and exert an immediate effect, while penicillin is effective only on actively multiplying microorganisms. Evidence has been presented in support of the fifth possibility in the case of penicillin-streptomycin and Terramycin-bacitracin combinations.

Klein has taken the view, and supported it by strong experimental evidence, that synergism occurs only when the microorganism tends to become resistant and is related to the inhibition by the second drug of resistant microorganisms surviving the action of the first. It follows that when resistance does not develop with some facility, synergism commonly does not occur, and some degree of antagonism may be observed. The consequences of combining drugs are dependent upon the drugs, upon the strain of microorganism, and upon the conditions under which the organism is subjected to the action of the drugs. On the basis of present information, then, the activity of combinations of drugs is more or less unpredictable, and this is in accord with the observations of many workers. The considerable uncertainty as to the indication for combined therapy may, then, be resolved by the requirement that the microorganism show a significant increase

in resistance to the chemotherapeutic agent. either promptly in acute disease or ultimately in chronic infections. Obviously, each of the combined drugs must have some activity against the microorganism, and they must differ from one another in their modes of action in such a way that cross-resistance does not develop. It is quite clear that as vet no rationale has been formulated for combined therapy of infections with microorganisms that do not become resistant with reasonable facility, e.g., infections with pneumococci, Str. pyogenes, and others, and the fundamental distinction must be made between infections in which resistance is and is not a factor.

Other applications. A number of the antimicrobial substances have useful applications other than in the treatment of infectious disease. The most important of these are the use of antibiotics as feed supplements and in food preservation. In the first instance it is established that the inclusion of small amounts (2 to 50 ppm) of antibiotics in animal feeds results in more rapid growth of domestic meat animals, increased hatchability of fertile eggs, and other indications of an enhanced nutrition. and such supplemented feeds are widely used. The nature of this effect is not clear.97 It is thought by some to be a result of increased vitamin synthesis by the intestinal flora. The traces of antibiotics persisting in foods of such animal origin are apparently not significant, though there may be sufficient activity in eggs to prevent the growth of bacteria for experimental purposes in the embryonated hen's egg.

The treatment of freshly dressed meats such as fish and fowl in dilute solutions of broad-spectrum antibiotics has a preservative effect such that they stay fresh appreciably longer, and these substances may well have broad application in food preservation.16, 20, 114 Chlortetracycline has been approved for this purpose in a concentration of 7 ppm; it is destroyed when the food is cooked.

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MICROBIAL VARIATION AND GENETICS

Prior to the development of methods of isolation of bacteria in pure culture, i.e., as clones, and the study of such isolates, these microorganisms were regarded as closely related or identical but of highly variable morphology. This view reached its zenith in 1877 with Nägeli, who postulated but a single species to which all bacteria belonged. This early concept of extreme pleomorphism was almost, but not quite, shattered by the study of bacteria in pure culture and the consequent development of a strict monomorphism by Koch and others which admitted substantially no change in the characteristics of bacteria in pure culture. It subsequently became clear that a more realistic view is a compromise between these two extremes, i.e., bacteria in pure culture have a continuing identity which is subject to variation within limits, although as yet these limits cannot be rigidly defined. Further, as the viruses have become better known, it has become evident that these forms too are subject to substantially the same kind of variability as the bacteria.

In a consideration of this aspect of microorganisms, it is essential to have clearly understood the reference point, or norm, from which deviations occur. Such a norm is difficult, if not impossible, to define in any absolute way. There is a tendency to assume as the "normal" the character of a microorganism occurring under conditions of man's devising, such as the properties of a microorganism growing or grown under laboratory conditions. One of the most obvious examples of the potential fallacy in such an assumption is the varied morphology of the symbiotic nitrogen-fixing bacteria of the genus Rhizobium between the regular,

rod-shaped form occurring on laboratory culture mediums and the so-called bizarre forms observed in its natural habitat, the nodules of leguminous plants, which is illustrated in the accompanying figure. In a very real sense there is no "normal," but rather many base lines which can be defined only in the context of the particular conditions under which the microorganisms have been grown and, to complicate matters further, often its past history. Any deviation, then, must be defined in terms of the reference point arbitrarily taken.

THE "UNIQUE" FEATURES OF MICROORGANISMS

In many respects microorganisms are seemingly unique among living things, and in a general sense this "uniqueness" is of three kinds. One is a consequence of their extremely small size, which has been touched upon earlier in relation to the morphology and growth of these forms. The accompanying simplicity of structure necessitates the use of differential criteria of a more subtle kind than those of comparative anatomy applicable to higher forms, viz., physiological and immunological properties. It is obvious that when a microorganism is so characterized, variability consists of deviation from some base line in these respects. Because such criteria are not usually applicable to higher forms, the impression is produced that the microorganisms are somehow "different." This is clearly fallacious as indicated by studies on the physiological genetics of higher forms and by the parallelism between the immunological relation of, for example,

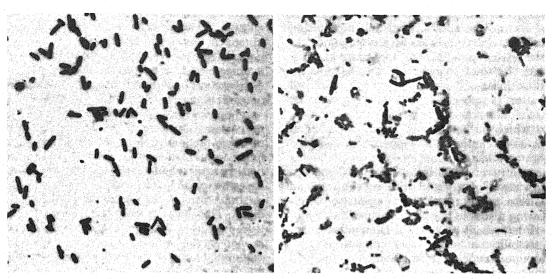


Figure 49. The morphology of Rhizobium. Left, a smear from a pure culture on laboratory mediums; note the regularity of the "normal" bacillary morphology. Right, smear from a clover root nodule showing swollen, branched, and coccoid forms, the so-called bacteroides. Fuchsin; × 1050.

the serum proteins of mammals and their phylogenetic relationships based upon a comparative anatomy (Chap. Thirteen).

The microorganisms share with other cells the essentially abstract environment in which factors such as exaggerated forces of surface tension and the predominance of interfacial phenomena at near-molecular or macromolecular dimensions play a large part. They differ from the differentiated cells of higher forms, not only in being smaller, but more significantly in being independent, complete units. As such they are not protected from marked variation in the immediate environment by the other components of a multicellular structure or by extensions of the structure such as regulatory mechanisms of homeostasis present, for example, in the mammal. Thus the directive mechanisms that maintain the integrity of the microorganism are not far removed from a variable external environment, and it might be anticipated that their functioning could be altered by extracellular influences. Such differences between microorganisms and other living things are, of course, a matter of degree rather than kind.

A second kind of seeming uniqueness derives from the extremely rapid rate of reproduction of the microorganisms. Whether it is a matter of environmental selection of the fittest from a mass of pre-existing heterogeneous individuals or of mutants conforming more closely to the requirements of a particular environment, variant forms appear

relatively rapidly. This might be taken to suggest that the microorganisms are unusually facile in this respect or, from another point of view, are relatively unstable with respect to at least certain of their characteristics. When the period of 30 years, generally taken to represent a human generation, is contrasted with that of 20 minutes for a rapidly growing microorganism, it becomes clear that the ready adaptability of microorganisms may be accounted for in large part by rates of reproduction. For example, mutation rates of bacteria are of the same general order as those demonstrable in more complex forms such as Drosophila, even though they appear much more rapidly in terms of elapsed time.

Finally, in contrast to many higher forms, the individual microorganism is not characterized except in a limited way, as with respect to size and shape of the individual cell. The statement that Bacillus X ferments such-and-such a sugar is not literally true. More accurately, it should be said that the sugar is fermented in the presence of a mass of many millions or hundreds of millions of the microorganism. This is not quibbling, but is rather, a recognition of the limitations imposed by the practical necessity for studying microorganisms as large populations, and recognition of this fact is of primary importance to an understanding of the mechanisms operative in microbial variation.

The properties of microorganisms must, then, be studied in terms of population biology. In the population, the individuality of its members is merged and disappears, and the characteristics of a population, expressed as statistics, are both more and less than the total character of the discrete entities that make it up. This is often a difficult matter to appreciate, for man reacts as an individual and conceives of the population to which he belongs as largely a matter of his own relation to it. Tippett has suggested that arriving at a concept of a population as an entity in itself is analogous to the appreciation of a fugue with its contrapuntal pattern as contrasted with the ease of following a simple harmony or tune.

For present purposes it is significant that variability is an inherent characteristic of populations; in fact, if each of its component entities were identical with all the others, it would lack such distinguishing characteristics as, for example, mean or median size. So far as is known, living organisms are seldom if ever precisely identical, and a

microbial population, such as a bacterial culture, is no exception but is seemingly homogeneous because it is studied as a population and individual differences are averaged out. Microbial variation is, then, a change in population character from which one infers causality on an individual basis.

It will be clear from the foregoing that microorganisms are not unique in the sense of being fundamentally different from other living forms. One may expect, therefore, to find that microbial variability has the same bases as variability of higher forms, although in some cases its expression may appear to differ. This has not only been found to be true, but, as will appear, study of variation in microorganisms has both broadened and pinpointed genetic mechanisms to an extent not possible in the study of higher organisms.

Here variation will be considered under two general heads, the kinds of variation observed, and the mechanisms operative in such variation.

Observed Variations of Microorganisms

Microorganisms have been found to vary, sometimes widely, in all the characteristics made use of in their differentiation and identification. These include morphology, both macroscopic and microscopic; physiological properties, including ability to produce disease; and immunological character. Changes may appear suddenly, i.e., in a single subculture, or they may become apparent only gradually, as in aged cultures or over many transplants or animal passages and some hundreds of generations. The rapidity of appearance, however, cannot be taken to have fundamental significance and, in fact, in many instances is subject to experimental manipulation. Variants may appear apparently spontaneously or may be seemingly induced, as by making the culture medium mildly and specifically toxic by the inclusion of lithium chloride, antiserum. antibacterial substances, and the like, or by making it selective so that the expected variant is given enhanced survival value as, for example, by including a sugar in the medium in attempts to isolate fermenting variants of a nonfermenting parent strain. In fact, in practice the single most potent factor in the demonstration of microbial variants is the selective nature of the environment, which may be either inadvertent

or planned. Without this factor the relatively rare variant or mutant cannot achieve the numerical status necessary to give an observable change in the population of microorganisms.

In any case, the variation is observed as a population characteristic in that the variant character of the individual cell is found in subculture, and the inference regarding the individual cell rests upon the assumption that the variant character breeds true for at least this long.

MORPHOLOGICAL VARIATION

Variation in the morphology of bacteria may be considered under two general heads: colonial morphology or that of masses of cells, and the morphology of individual bacterial cells.

of bacterial dissociation. A remarkable type of bacterial variation, whose most obvious outward manifestation is a change in the type of colony formed on semisolid mediums, was observed by Baerthlein in Germany (1918), Arkwright in England (1921), and de Kruif in the United States (1921). The phenomenon was termed by de Kruif

bacterial or microbic dissociation, a term which has, in spite of certain undesirable features, been generally adopted.

Colonial morphology. The ordinary laboratory culture, particularly old broth cultures, when plated out on an agar medium develops into two kinds of colonies: one is smooth (S), round, convex, and shining; the other is rough (R), irregular, flattened, and wrinkled. In addition to these obviously different extreme colony types, all degrees of intermediate (SR and RS) types may usually be found. The transformation of the S form, usually regarded as the "normal," into the R type is, as indicated by the presence of intermediate types, probably a gradual process. The smooth form of the great majority of bacteria, while breeding true for a number of test tube generations, shows a constant tendency to change to the rough type; one of the consequences of this tendency is that most stock laboratory cultures contain a large proportion of roughs, and sometimes such cultures are completely lacking in smooth forms.

A third kind of colony, the M or mucoid, is also commonly recognized, characterized by a slimy, mucus-like consistency and appearance. The mucoid character may be associated with the S or R colonial types to give mixed or intermediate forms, but it is thought by many that S is intermediate between M and R and that the succession is $M \rightleftharpoons S \rightleftharpoons R$. In individual cases other

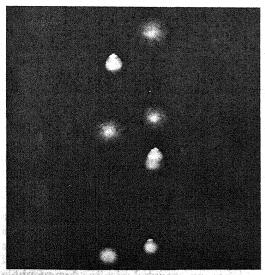


Figure 50. Smooth and rough colonies of Salmonella typhi. Culture on nutrient agar inoculated from an old broth culture, × 3.

colonial forms may be observed; the hemolytic streptococci, for example, occur in a form designated "matt" by Todd and the British workers which has a ground-glass appearance when viewed by reflected light. Still another form, the dwarf colony or D type, may be produced by experimental manipulation and is sometimes isolated from the natural habitat of the microorganisms. Colonial differences, often very slight in character but frequently associated with virulence and other physiological properties, are apparent when the culture is examined under low magnification by oblique, transmitted light as described by Henry.⁴⁴

The transformation of S to R, though occurring in ordinary cultures, may be hastened by a number of procedures, such as the inclusion of lithium chloride or anti-S immune serum in the medium in which the organisms are cultured. Smooth cultures lysed by bacteriophage give rise when incubated to a secondary growth which often consists entirely of R forms. The change from R to S is considerably more difficult, and the rough forms show little tendency to revert under ordinary cultural conditions. The reversal may, however, be accomplished in most cases, one of the most effective methods being the cultivation of the R form in the presence of anti-R immune serum. Reversion of the R type may also be brought about by animal passage.

Properties of S and R variants. The bacteria which make up the smooth and rough colonies differ from one another in a number of characteristics, some of which are usually regarded as of deep-seated biological significance.

The distinctions between the S and R types given in the accompanying tabular form represent a generalization to which there are a number of exceptions. For example, what is usually regarded as the "normal" form of the anthrax bacillus is the rough form, and in this case the organism is virulent in the rough form but avirulent in the smooth form. Similarly, the "normal" virulent form of the hemolytic streptococci is finely granular rather than smooth, and the avirulent form which arises from it has lost its typespecific antigen but is smoother than the virulent form. In spite of these and other exceptions, the differences noted above hold true in general.

Cellular morphology. Closer inquiry into the differences between S and R bacterial types indicates that the relation between

Contrasting Properties of S and R Varieties

S TYPE

R TYPE

Colony usually smooth and viscous Pathogenic forms usually virulent Forms stable suspensions in salt solution

Encapsulated forms have capsules Antigenically "complete"

Colony often dry and granular and may be larger Pathogenic forms often relatively avirulent Spontaneously aggregates and settles out in granular deposit from suspension in salt solution Capsules diminished or absent Often show loss of antigens, sometimes with the appearance of "new" antigens

colony form and certain other characters is not in the nature of a casual correlation but rather the result of an intimate association. The association between capsules and virulence has been pointed out previously, as has been the role of the capsule in giving the colony of encapsulated bacteria in a slimy, viscous consistency. It is not unreasonable to expect, then, that a bacterium which lacks a capsule is also avirulent, or relatively so, and that its colonies will be dry and possibly wrinkled in appearance. The loss of a capsule likewise alters the nature of the bacterial surface: the absence of carbohydrate material allows the lipids present in the cell membrane to determine its character; in consequence, it becomes hydrophobic rather than hydrophilic, and the organisms tend to clump and settle out of salt solution or the nutrient broth of a culture. The granular consistency of many of the rough colony types is accounted for, in part, by postfission movements of the cells. The smooth forms of some of the intestinal bacilli separate after fission and slip to lie side by side with one another. The cells of the rough forms, on the other hand, tend to remain attached after fission with the formation of chains which bend at sharp angles here and there. The development of bizarre shapes by some of the rough forms may possibly be a result of the absence of capsular material and change in the nature of the cell surface. The nature of the association between roughness and reduced biochemical activity is unknown.

Induction of dissociation. Experimentally, the inclusion of antiserum to the S or R form will frequently result in the appearance of the other form; the occurrence of avirulent R forms of pathogenic microorganisms isolated from chronic carriers of the infection is presumed to be a consequence of the effect of antibody developed to the virulent S form.

Most often the change, especially the $S \rightarrow R$, occurs in old liquid cultures and is an effect of accumulation of metabolites. The role of metabolites in the $S \rightarrow R$ dissociation has been strikingly illustrated by the studies of Braun and his colleagues^{24, 36} on Brucella. Smooth strains cultured in synthetic medium containing asparagin as the sole source of nitrogen multiply in the usual way, but after some days of incubation are replaced by R forms. The occurrence of R forms coincides with the accumulation of d-alanine, which is specifically toxic to the S but not the R form. The $S \rightarrow R$ shift is prevented by including sodium pyrophosphate in the medium. This substance forms complexes with manganese and magnesium, trace elements that act as catalysts in alanine formation; the effect of pyrophosphate is in turn antagonized by the addition of manganese or magnesium. Smooth virulent forms, differing from the original smooth type in that they are resistant to alanine, may also be isolated. It is of considerable significance that the effects of amino acids may occur in vivo also. For example, in the case of Salmonella typhimurium, virulence for the mouse was greatly enhanced by administration of threonine to the animals; a more highly virulent, threonine-resistant variant, occurring as less than 1 per cent of the original inoculum, was selected in vivo.78

Variation in cell structures. As indicated above, colonial morphology is in part a function of the morphology of the individual cells making up the colony, and one of the most important factors in the differential morphology of the S and R variants is the occurrence of hydrophilic polysaccharide on the surface of the cell. As pointed out elsewhere, this frequently takes the form of a layer or morphologically demonstrable capsule on the outer surface of the cell.

Capsules. Capsule formation or, from the

physiological point of view, the synthesis of capsular substance usually of polysaccharide but sometimes of polypeptide nature, is very common among the bacteria, but a morphologically demonstrable capsule is not always apparent. Capsule formation is dependent in large part on the environment, and the most favorable medium in the case of pathogenic bacteria in which capsule formation is associated with virulence is the body of the susceptible animal. The anthrax bacillus forms a heavy capsule in the infected animal, but there is little or no evidence of a capsule when the bacilli are cultivated on laboratory mediums. The pneumococcus, likewise heavily encapsulated in the infected animal, also forms a capsule in artificial mediums, but the capsule is best developed when the medium is enriched with blood or serum and carbohydrate. Among the pathogenic forms. capsule formation tends to diminish with continued cultivation on artificial mediums. probably because these furnish a somewhat less than optimal environment, and is restored by animal passage of the bacteria.

The heavily encapsulated saprophytic bacteria, such as the dextran- and levulanforming cocci of the genus Leuconostoc, show no tendency to loss of capsule formation in culture provided that carbohydrate is supplied, and it seems probable that the artificial medium is more nearly optimal than it is in the case of the more fastidious pathogenic bacteria. In general, then, capsule formation is determined in large part by the environment, may be a quantitative matter, and is reversible.

Capsule formation may be specifically inhibited, however, the most effective means being the inclusion of antiserum to the capsular material in the culture medium. The loss of capsule formation under such circumstances, or when it is relatively complete by thorough prolonged culture on artificial mediums, results in the S-R dissociation, and the lack of a capsule is characteristic of the rough variant. As indicated above, this loss is also reversible, though frequently only with considerable difficulty. Whether it is to be regarded as basically different from a gradual diminution in the amounts of capsular substance synthesized through continued cultivation in a less than optimal environment is not clear.

Flagella. The occurrence of flagella on motile bacteria is also subject to variation. There appears to be little or no variation with respect to numbers or position of flagella, but

their presence or absence is variable. Nonmotile variants of motile bacteria are occasionally, though not commonly, observed, and these appear to be stable forms with little tendency to reversion to the motile form. In the case of very actively motile bacteria such as Proteus, colonial morphology is affected by motility in that the motile bacteria "swarm" and the growth spreads in a thin film over the surface of an agar medium, while the nonmotile variants form discrete colonies.

The formation of flagella is subject to environmental influence also. Actively motile species kept in stock culture on laboratory mediums tend to lose their motility, and potentially motile bacteria may be motile when cultivated at one temperature but not at another. The inclusion of antibacterial substances such as phenol in the medium in toxic but nonlethal concentrations inhibits the formation of flagella by bacilli of the Salmonella group; in fact, one method of obtaining these forms free from flagella and the antigenic substances associated with them is culture on nutrient agar containing 0.1 per cent phenol.

Spores. The spore is a consequence of the aggregation of cell substance within a spore case during spore formation. It is this process rather than the formation of a structure of the vegetative cell that is subject to variation. Like capsule formation, it is essentially a physiological process but with morphological consequences. Spore formation is affected by environmental factors, and asporogenous variants which breed true also occur. In the case of the anthrax bacillus, for example, spores are formed only when the bacteria have access to free oxygen: only vegetative forms are found in the infected animal until the bacilli are exposed to air by tissue decomposition or autopsy and then spore formation occurs. Similarly, spores are formed most rapidly at 32° C., less so at 37° C., and not at all when the culture is incubated at 42° C. The inhibition of spore formation by the anthrax bacillus by high temperatures is a temporary one in that when the temperature of incubation is reduced spore formation again occurs, but if cultures are maintained at 42° C. for many transfers the ability to form spores is apparently permanently lost. In general, however, spore formation is a relatively stable character.

L forms. 57 A kind of variation known as

L variation was first observed by Kleineberger in cultures of Streptobacillus moniliformis (one of the causative agents of rat-bite fever) and subsequently by other workers, especially Dienes, in cultures of a variety of bacteria. The bacteria become more transparent and stain with difficulty or form granules which unite and grow into the amorphous bodies of variable size known as L bodies. These bodies may multiply or give rise to bacterial cells morphologically indistinguishable from the parent strain.

The significance of these variants is uncertain. In morphology they are similar to the pleuropneumonia microorganisms (Chap. Twenty-seven) and appear to be etiologically related to polyarthritis of rats. Whether or not the L bodies are variants of common bacteria and have more than a superficial relation to the pleuropneumonia organisms is uncertain. It is also possible that these bodies represent naturally occurring protoplasts of greater stability than those prepared by enzymatic digestion of the cell wall (Chap. Three); if so, they could be regarded as bacterial variants which are unable to form a complete cell wall structure. 63, 108, 111

Aberrant forms. The occurrence of aberrant or "abnormal" forms of bacteria, differing from the presumably normal morphology of recent isolates and cultures on favorable mediums, is commonly observed. In the earlier literature these are called involution forms and regarded as primarily degenerative in character. While this term is applicable in some cases, it is not necessarily appropriate in all, for some kinds of bacteria show varied morphology as a rule rather than an exception.

The tendency to varied morphology is in part an effect of the environment on the cell and in part a function of the relative rigidity of the cell structure. For example, a transitory alteration in cell shape may be produced by culture of bacteria in toxic, but not lethal, concentrations of substances such as NaCl or certain antibiotics. The distortions produced in the first instance are probably in large part an osmotic effect, and in the second seem to be a consequence of inhibition of processes of cell division while synthesis of intracellular substances continues, giving rise to swollen forms. Or, the inclusion of surface tension depressants in the culture medium frequently results in the growth of bacilli in the form of long filaments and



Figure 51. Involutional forms of the typhoid bacillus. Note the filamentous forms and elongated rods mixed with the typical forms. What appear to be buds on the filament are probably adjacent cells. The poor resolution at these high magnifications is apparent. Fuchsin; × 3500.

similar phenomena. Such aberrant forms are usually viable and on subculture produce normal progeny.

Bizarre forms are also commonly observed in old cultures, particularly in liquid mediums. This is probably a consequence of the presence of relatively large numbers of dead and dying cells and possibly also of the toxic effects of accumulated products of metabolism. When the bacterial cell is dead or dving, its structure begins to break down with autolytic changes. The protoplasm becomes granular and may escape into the surrounding medium, coccoid forms of bacilli and other swollen forms result from the breakdown of the osmotic barrier, etc. For example, the pneumococcus produces peroxide as a terminal product of respiration, but no catalase, so that peroxide accumulates to lethal concentrations, killing the cells. These bacteria contain intracellular proteases, and autolytic changes set in rapidly. One of the first indications of this is a loss of the ability to retain the Gram stain, and even in 24-hour cultures many cells are found to be gram-negative. Similarly, the cholera vibrio is a fragile cell and may be broken up mechanically more readily than most other bacteria; it also contains a powerful intracellular protease; and it is not surprising that it is notorious for its

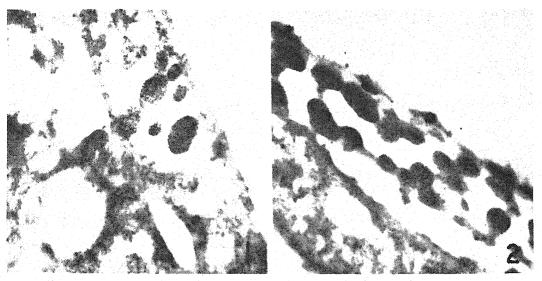


Figure 52. The varying morphological types of feline pneumonitis virus during one-step growth in chorioallantoic ectoderm. Left, the ovoid granulated forms occurring at 15 hours, shown at point 1 on the growth curve in Figure 53. Right, the smaller forms at 20 hours, at point 2 on the growth curve. Electron micrographs, × 15,000. (Litwin.)

morphological variability. Such cells are often dead, in that they do not reproduce on subculture and may be regarded as involution forms.

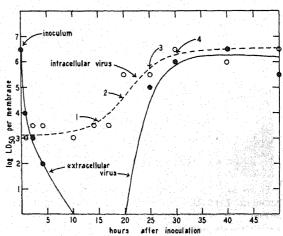
Still other bacteria, notably forms such as Bacteroides and the pleuropneumonia organisms referred to above, seem to be so fragile in structure that they do not occur as a single or predominant morphological type. The Bacteroides are rod-shaped but occur in filamentous and other varied forms, while the pleuropneumonia organisms have a widely varied morphology, including coccoid forms, symplastic masses, and ring forms. Such varied morphology under standard

conditions of culture is, however, the exception, and the great majority of true bacteria, or Eubacteriales, are remarkably constant in this respect.

Microbial life cycles. The occurrence of morphological variation among the bacteria inevitably raises the question of whether morphological variants are random events or have a deeper significance as expressions of cyclical events in the life history of the microorganism.

There is no doubt that simple sequential developmental changes expressed morphologically occur. Obvious examples are the spore-vegetative cell sequence occurring

Figure 53. One-cycle growth curve of feline pneumonitis virus in chorioallantoic ectoderm. The dotted line represents intracellular virus, and the solid line that occurring free. The points labeled 1, 2, 3, and 4 on the intracellular virus growth curve are the stages in the cyclic development of the virus illustrated in Figures 52 and 54. (Litwin.)



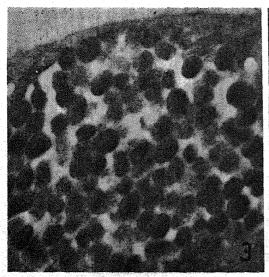
during the growth of spore-forming bacteria, the sequence of morphological types occurring during the growth of a culture and designated cytomorphosis, and the successive zoogleal and monad or swarmer stages of Nitrobacter. Such sequential changes can hardly be taken as expressions of life cyles such as, for example, that of Plasmodium.

The evidence for the occurrence of bacterial cyclogenies is substantially a matter of the observation of cells of varied morphology, in old cultures or similar unfavorable circumstances, and their arrangement in an order that may be taken to represent a sequential relation. The relatively constant morphology of most kinds of bacteria under defined conditions of culture thus becomes but one stage in the life cycle of the bacterium which is maintained to the exclusion of other stages by the environment. The fallacy lies in the arrangement of morphological variants, for it is necessarily subjective rather than objective, and the evidence is less than unequivocal. The possibility of the occurrence of successive developmental stages is not, of course, eliminated, but in any consideration of this kind it must be kept in mind that the organization of the microbial cell is largely on, at most, a macromolecular level, and variation need not have morphological expression. In fact, the great bulk of variations are physiological (see below) and are only rarely associated with morphological changes.

Virus replication cycles. As indicated elsewhere (Chap. Four), replication of the viruses and virus-like agents is characterized by the occurrence in time sequence of successive, differentiable stages. For the most part these stages are separable by immunological and infectivity criteria, and, with rare exceptions, notably the protein head structure of incomplete coliphage, these stages have no morphological counterparts.

Among certain of the larger viruses, however, varied morphology of the virus particle may be observed. The differentiation of the intracellular matrix or inclusion body into particles of poxvirus, heterogeneous with respect to the amount and distribution of electron-dense, presumably nucleic acid, material within the particle has been described elsewhere (Chap. Four). These forms occur admixed within the host cell and, while they may be arranged in an order, the arrangement is as yet arbitrary rather than determined by time or other objective factor.

Members of the psittacosis-lymphogranuloma group have been studied in most detail in this respect, for it was apparent quite early that morphologically different particles occur



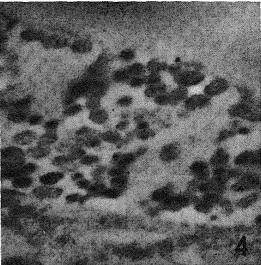


Figure 54. The varying morphological types of feline pneumonitis virus during one-step growth in chorioallantoic ectoderm. Left, the smaller forms have increased in number, corresponding to point 3 on the growth curve shown in Figure 53. Right, mature forms occurring at the end of the growth cycle at 30 hours and showing two morphological types, the smaller dense-centered bodies and the larger granulated forms. Electron micrographs, × 15,000. (Litwin.)

during the course of the reproductive cycle. Their occurrence in an orderly sequence was suggested by studies with the light microscope, 112 and subsequently they were more precisely defined by electron micrography. The growth cycle of feline pneumonitis microorganism in ectodermal cells of the chorioallantois of the embryonated hen's egg is completed in about 30 hours.64 By 15 hours the spherical elementary body, about 200 m μ in diameter, has increased in size to 500-700 m μ and contains an increased amount of electron-dense material: at this point the growth cycle corresponds to the lag phase of bacterial growth. At 20 hours the bodies are somewhat smaller, 500 mμ, somewhat increased in numbers, and the vesicle within which they are contained in the host cell is larger. By 25 hours growth is exponential, the bodies are transitional in size, ranging from 200 to 500 m μ , and the vesicle practically fills the host cell. The end of the cycle is reached by 30 hours, with the appearance of two kinds of mature particles, the one a small spherical body about 200 m μ in diameter and containing a central body of electron-dense material, and the other a larger ovoid particle, 300 × 500 m μ , which is reticulate or granular in structure. The analogy to the successive stages of bacterial cytomorphosis during growth is striking, but, since the microorganisms of this group are set somewhat apart from viruses, it does not follow that virus growth cycles in general might be expected to be reflected in such a succession of morphological types.

In summary, there is as yet no convincing evidence of the occurrence of bacterial life cycles other than the cytomorphosis occurring during the population growth cycle. The virus-like agents of the psittacosis-lymphogranuloma group show a similar pattern of successive morphological types of particle, but the clearly apparent stages in the developmental cycle of viruses are characterized by immunological methods and infectivity.

PHYSIOLOGICAL VARIATION

Variations in the so-called physiological characteristics of a microorganism are not fundamentally different from morphological variations, in that the latter involve the synthesis of cell structures, but in practice they

are taken to be those which do not necessarily have a morphological expression. These include the ability of a microorganism to decompose sugars and other substrates with the appearance of characteristic end products (the biochemical properties of microorganisms), their nutritive requirements, susceptibility to infection with bacterial viruses, immunological character, and, in the case of pathogenic forms, virulence.

All of these may be equated on the common basis of the synthetic abilities of the microorganism. The decomposition of various substrates is obviously dependent upon the elaboration of the appropriate enzymes, and nutritive requirements are an expression of synthetic abilities in that a substance required for growth (in the sense that it must be supplied preformed) is one that the microorganism cannot synthesize. Susceptibility to infection with bacterial viruses is dependent, in part at least, on the presence of appropriate receptors in the cell wall structure, a product of synthesis. Similarly, immunological character is an expression of the antigenic specificity of components of the cell substance of the microorganism. or the products of its metabolism, both the result of synthesis. The nature of the virulence of pathogenic microorganisms is not fully understood, but it is indicated that the ability to produce disease is in large part a consequence of the production of toxic substances, ranging from potent exotoxins to enzymatic or kinase-like activities that facilitate invasion of the tissues of the host (Chap. Nine). Such substances are also products of microbial synthesis.

The identity of a microorganism in all these respects is thus referable to the operation of directed control mechanisms within whatever limitations are imposed by the environment. It follows that microbial variation arises from alterations in genetic control mechanisms and/or their functioning within the microcosm of the environment to give phenotypic expression of genotypic character.

ENVIRONMENTAL SELECTION

The demonstration of microbial variants almost always requires the utilization of a selective environment such as a culture medium that may be differential as well as selective or an experimental animal or its

equivalent in the case of virulence studies. This is because ordinarily only a minute portion of the microbial population differs from the parent strain, and its emergence from the population to recognizable proportions is usually dependent upon an environment which is more favorable to the development of the variant than of the parent strain.

This approach is applicable directly in the case of variants in which the change is positive, in the sense of the acquisition of the ability to ferment a sugar, to produce infection in an experimental animal or the development of resistance to an antimicrobial substance. For example, when a substrate which the parent strain is unable to utilize is present in a culture medium, the variant which is able to decompose it may be able to outgrow the parent strain. If the substance is a sugar it may be incorporated in an agar medium together with an indicator. such as an acid-base indicator or Schiff's reagent, so that the colonies of the variant will be colored while those of the parent strain are not. Or, when the parent strain requires, for example, an amino acid for growth, variants which are able to synthesize the amino acid grow in a medium lacking it while the parent strain does not. In the same way, a culture medium may include an antimicrobial substance, such as an antibiotic, which prevents the growth of the parent strain but not that of a resistant variant, or after inoculation with bacteria the medium may be inoculated with bacterial virus which inhibits a susceptible parent strain but not a resistant variant. Similarly, virulent pathogenic microorganism grows readily in the tissues of the host, while the avirulent form fails to establish an infection and is destroyed by host defense mechanisms.

Variations involving losses require a different approach in that the growth of the parent is not suppressed in the absence of the substrate while that of the variant is. Consider a parent strain which does not require tryptophan and a variant which does; both will grow on a medium containing the amino acid, but only the parent strain on a medium lacking it. Individual colonies may be subcultured from the complete medium and tested, but this procedure is laborious and, for the isolation of such variants on any scale, an indirect method is required.

In this connection advantage may be taken of the fact that penicillin affects actively multiplying bacteria to a much greater extent than those which are not multiplying. An agar medium deficient in some nutrient, such as an amino acid, is inoculated and incubated for sufficient time to allow active multiplication of those cells which do not require the amino acid. It is then treated with penicillin to selectively destroy the actively multiplying cells, the activity of the penicillin is neutralized, and the inoculated medium is overlaid with agar medium containing the amino acid, or other substance, not present in the first medium. The cells which were unable to multiply in the absence of the substance in the first instance, and so were not affected by the penicillin treatment, grow out in the second medium.

ADAPTATION103

For present purposes the term adaptation may be interpreted broadly as covering all microbial variations which are consistent with an increased physiological compliance with the demands of the selective environment. The adaptive response is of two kinds. One of these is actually a response to the environment in that it is induced, more or less directly, by an environmental factor. The other is not a response in the literal sense, and its adaptive character is a consequence of the appearance of a random mutant which, purely by chance, has greater survival value than the parent from which it was derived. In a general way, the induced variation usually develops slowly and the adaptation seems to be continuous, while spontaneous mutants appear abruptly and the adaptation which is dependent upon a series of mutations occurs as discrete steps. It is often not possible to distinguish between these two kinds of adaptation, and both may be involved in what seems to be a single adaptive process.

ATTENUATION

The term attenuation is ordinarily loosely used to mean altered virulence of a pathogenic microorganism. It may indicate a simple loss of ability to produce disease in a general sense or a reduction in virulence for one host species accompanied by an increase in virulence for another host species.

Loss of virulence. In general, most pathogenic bacteria tend to lose virulence when kept in culture on artificial mediums. For example, pneumococci rapidly lose virulence. for the mouse and in even a very few transfers on enriched mediums the minimal lethal dose increases by several hundred-fold. Staphylococci, however, retain their virulence over many transplants on artificial mediums but eventually become relatively avirulent. A few bacteria, such as the anthrax bacillus, seem to retain virulence almost indefinitely, but this is not common. Such losses in virulence are general in character in that the bacteria become less virulent for a variety of experimental animals and presumably arise as a consequence of adaptation to an environment other than that of the animal body.

The artificial environment may be deliberately made somewhat toxic in order to bring about such changes. Simple aging in the case of Pasteur's cultures of the fowl cholera organism was sufficient to reduce virulence so that a fatal infection was not produced, and the attenuated strain of bovine tubercle bacillus known as BCG was carried on a bile-containing medium until its virulence was apparently completely lost.

Loss of virulence may be accompanied by changes in other characteristics such as marked diminution in capsule formation by the pneumococcus, a loss of golden pigment by Staphylococcus aureus and somewhat less fastidious nutritive requirements. In many instances the change is dissociative in nature, and loss in virulence is associated with a shift from the smooth form. In most bacteria, of course, virulence is markedly reduced by the S-R dissociation, and a change of this kind may occur in vivo, presumably under the influence of antibody; for example, the typhoid bacilli excreted by chronic carriers are often rough, avirulent forms.

Animal passage. Virulence may often be restored by animal passage, e.g., successive and repeated infection and re-isolation of the bacterium through a series of experimental animals. It cannot be concluded that the microorganisms are individually altered in the restoration of virulence by animal passage, and it is more probable that the animal acts as a highly selective screen, separating out those few bacteria in the inoculum which multiply most rapidly in the tis-

sues. The restoration of virulence is usually accompanied by a restoration of other characters associated with it such as capsule formation.

Modification of virulence. The virulence of a microorganism may be modified by passage through another host species, and sometimes, though not invariably, virulence for the original host species is reduced. The classic example of attenuation by animal passage is that of Pasteur's attenuation of the rabies virus by adaptation to the rabbit brain. The original strain from dogs, "street virus," kills rabbits in about two weeks following subdural inoculation, after some 20 passages it kills in eight days, and after an additional 20 or more passages the period may be reduced to seven days but cannot be reduced further. This "fixed virus" will not produce rabies on subcutaneous inoculation and so may be used as an immunizing agent. Such attenuation or adaptation to a new host is very common among the viruses and includes the adaptation of a variety of viruses to the chick embryo, the adaptation of vellow fever virus to the mouse brain, etc. For example, on primary isolation in the embryonated hen's egg, influenza virus forms relatively little hemagglutinin for the chicken erythrocyte, but after a few passages hemagglutinin is formed in abundance. This has been designated the O-D variation of influenza virus and represents an adaptation to the new host tissue. This virus can be further modified to, for instance, the mouse lung, or its naturally occurring pneumotropism altered by successive passage in the mouse brain so that, like the adapted yellow fever virus, it becomes neurotropic.

BIOCHEMICAL VARIATION

Variation in the biochemical properties of bacteria, such as fermentation of sugars, decomposition of proteins and amino acids, nutritional requirements, resistance to antibacterial agents, and the like, occurs with some frequency. Some of these develop more or less gradually on continued cultivation in selective mediums and resemble the process of attenuation of virulence.

Adaptation and training. Such variation in nutritive requirements is the basis for the general observation that the more fastidious pathogenic bacteria are frequently more difficult to cultivate on primary isolation and

enriched mediums are required, while, after being carried in culture for some time, they grow more rapidly and profusely and on somewhat simpler mediums. For example, on primary isolation Brucella abortus requires an increased carbon dioxide tension, but after a few subcultures this may be dispensed with. Similarly, the gonococcus and meningococcus require, on primary isolation, both an increased carbon dioxide tension and an enriched medium, usually a heated blood medium. After a few subcultures, however, it is no longer necessary to supply carbon dioxide, and eventually growth occurs in simpler mediums. The adaptive process may be initiated, or markedly accelerated, by culture of bacteria in mediums designed to select variants. Pathogenic staphylococci become less fastidious in their nutritive requirements when maintained in the laboratory as noted above, and variation in this direction may be accelerated by forcing the bacteria to grow in a medium that is made successively less and less complex. For example, the amount of a required amino acid present in a synthetic medium may be gradually reduced over successive transplants, until finally the microorganism no longer requires it. By such means fastidious staphylococci have been trained or adapted to grow in a simple medium containing only glucose and an ammonium salt. Similarly, typhoid bacillus strains requiring the single amino acid tryptophan may be trained to grow in its absence, and strains of dysentery bacilli capable of growing in a glucose-ammonium salt medium, supplemented only with nicotinic acid, may be adapted to dispense with the vitamin.

Ability to ferment carbohydrates may also be either acquired or markedly enhanced by continued cultivation in the presence of the substrate or by very heavy inoculation of highly specific mediums in which the substrate is the only nutritive material. Still other adaptations such as to changes in pH, increased incubation temperature, and the like, also occur but are usually not marked.

Discontinuous variations. Some microbial variants appear much more rapidly than those developing in the kind of adaptations described above, often occurring in the first culture on a selective medium. They are regarded as expressions of a sudden, discontinuous change, or mutation in the genic

sense, in the genetic apparatus of the microorganism.

Lactose fermentation. The classic example of this kind of variation is that shown by certain strains of coliform bacilli which are atypical in that they are unable to ferment lactose, but which throw off lactose-fermenting variants. This phenomenon was observed as early as 1907 by Massini, and the strains of coliform bacilli were named Bacterium coli mutabile.

The phenomenon is demonstrated by culture of the bacteria on an agar medium containing lactose together with an acid-base indicator which turns red in the presence of acid. The primary growth consists of white colonies of the nonlactose-fermenting parent strain, and on continued incubation a secondary growth of the lactose-fermenting variant appears in the form of red papillae on the white colonies. On subculture on the same medium, the lactose-fermenting variant breeds true, while the parent strain again produces white colonies on which red papillae appear. The enzyme produced by the variant, but not by the parent strain, is the adaptive enzyme β -galactosidase. As an adaptive enzyme (see below), it is produced only in the presence of an inducer, in this case lactose and certain related compounds, and the variant is characterized by the ability to form the enzyme and its potentiality is realized in the presence of the substrate. In genetic terminology, therefore, the genotype of the parent strain is lac- and that of the variant lac+, but both are phenotypically the same in the absence of inducer.

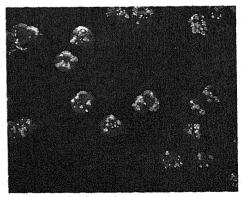


Figure 55. Colonies of E. coli mutabile on lactose agar. Note the lactose-fermenting papillae of the variant appearing on the nonlactose-fermenting colonies. (Parr.)

Other variations. Many other variants of this rapidly appearing kind are well known and have been studied extensively, especially variants resistant to chemotherapeutic agents (see below) and those requring essential metabolites such as amino acids and purines. These distinctive characters have been used as markers in studies of the genetics of microorganisms for they behave in a manner consistent with the assumption that they are determined by genes or their equivalent.

Analogous variations occur among the viruses also. The characteristics subject to variation are not physiological, since these agents apparently have no independent metabolism, but are characteristics such as plaque size and appearance and burst size of the bacterial viruses, and immunological changes, virulence, and cytotropism of animal viruses, of which influenza is the best known in this respect.

Variation of this kind cannot be dissociated from the adaptive processes described above. Such a variant, with some small advantage in growth rate over the parent strain in a selective environment, would displace the parent type more or less slowly to give the appearance of a slowly developing adaptive response. The microbial population tends to obscure the nature of the processes of variation, but it is likely that spontaneous mutation plays a significant part in adaptation.

RESISTANCE TO CHEMOTHERAPEUTIC AGENTS^{35, 82, 91}

The development of strains of microorganisms resistant to chemotherapeutic agents was observed as early as 1907 in connection with the parafuchsin therapy of trypanosome infections in mice. It has been observed since that microorganisms will become resistant to a variety of compounds, including aniline dyes (parafuchsin, acriflavine), aromatic arsenicals (Atoxyl, arsacetin, tryparsamide), aromatic compounds of antimony (stibenyl, Fuadin), suramin (Bayer 205), styryl quinoline derivatives, guanidine compounds, sulfonamides, and antibiotics.

Prior to the introduction of sulfonamides, therapy of infectious diseases was limited to the diseases caused by spirochetes and animal parasites. When sulfonamides, and within a few years antibiotics, became available, they became so widely used that the problems created by microbial resistance to chemotherapeutic agents assumed large proportions.

The facility with which resistant strains of microorganisms develop is a property of both the microorganism and the antimicrobial substance. For example, staphylococci become resistant to many kinds of antibiotics very readily, the gonococcus becomes resistant to sulfonamides but apparently less readily to antibiotics, while pneumococci and streptococci are only occasionally resistant to sulfonamides or antibiotics but not to a practically important degree. Conversely, it is characteristic of streptomycin that almost all kinds of bacteria become resistant to it, often very rapidly. Microbial resistance may be considered here from two points of view, that of natural occurrence and therefore inferentially in vivo, and that of experimental production in the laboratory or in vitro.

Resistance acquired in vivo. The development of resistance under natural conditions of use of antimicrobial substances for therapeutic purposes is expressed as an increased proportion of resistant strains among those found in disease conditions or in carriers. When the sulfonamides were first introduced into general use, they were effective in the treatment of the majority of cases of gonorrhea, but within a relatively short time, three or four years, only a minority of cases of this disease could be treated effectively with these compounds, and strains of gonococcus isolated from refractory cases were shown to be resistant. Subsequently the response to repository penicillin has become less satisfactory, and is considered to be a factor in the increased prevalence of gonorrhea.¹⁸ With the consequent decline in the therapeutic use of sulfonamides, there is now evidence that the prevalent strains are becoming more sensitive to sulfonamides, and these compounds may again be effective.113

Similarly, the proportion of staphylococcus strains resistant, first to penicillin, subsequently to tetracyclines and other antibiotics, and often showing multiple resistance, increased at a rapid rate as the antibiotics became generally available and widely used.⁸ There is evidence too that there is an appreciable sulfonamide resistance developing among the dysentery bacilli.¹⁰⁹

The occurrence of resistance is related to the frequency with which antibiotics are used. Staphylococcal resistance has been studied most intensively, is of the most practical importance, and may be used here as an example. Resistance to penicillin, the first generally used antibiotic, appeared first, and then resistance to tetracyclines and chlormaphenicol, and subsequently to newer antibiotics such as erythromycin. Chloramphenicol contains a nitro group (Chap. Six), and after a number of cases of aplastic anemia were associated with its continued administration, it was used much less commonly. Coincident with this, there was a decline in the proportion of staphylococcus strains resistant to it. This, and other evidence, such as that of reversal of sulfonamide resistance among gonococci, suggests that, as measured by the proportion of resistant strains, bacterial resistance in nature is not established permanently, but rather is maintained by continued general use of a chemotherapeutic agent. The tendency to reversion to sensitivity, coupled with the variety of chemotherapeutic agents now available, opens the way to circumventing the problems of effective chemotherapy. In Hammersmith Hospital in London, for example, it has been possible to reverse the tendency to increasing antibiotic resistance of staphylococci and reduce the frequency of staphylococcal infection by balancing the kind of chemotherapeutic agents used against the observed frequency of resistance, together with special attention to sterilization and prevention of cross-infection.9

While, as might be anticipated, resistant strains occur in greatest proportion in hospital populations, for the most part such resistant strains are not introduced by the patient, but rather infection of the patient with resistant strains is acquired from hospital personnel. The source of infection is most often carriers, usually nasal carriers, and the incidence of nasal carriers of resistant staphylococci among surgeons and surgical personnel may become high. It is not uncommon to find that a wound infection following surgery is one with staphylococci having multiple resistance which does not respond to treatment with the commonly used, and generally available, antibiotics. The problem so created has reached such proportions that bacteriological control of surgical personnel has become necessary in some instances. Such studies as have been made of the incidence of resistant staphylococci in the general population suggest a proportion of perhaps 20 per cent, though this may vary widely and be much lower. In contrast, the proportion of penicillin-resistant staphylococci in hospital personnel commonly ranges from 60 to 85 per cent in this country and in Western Europe. The general problem of the incidence and behavior of bacterial strains resistant to chemotherapeutic agents has been studied intensively.^{32, 52}

Origin of resistant strains. It is not completely clear whether resistant strains of microorganisms are pre-existing and make up a small, possibly minute, portion of all strains, or whether they arise for the most part in the individual undergoing chemotherapy. In the first instance, when the microorganism is primarily a parasite of man, the widespread use of a chemotherapeutic agent alters its environment to favor the survival of resistant strains. Under such circumstances it is to be expected that the proportion of resistant strains would increase.

It is well established, on the other hand, that resistant strains of microorganisms appear in persons originally infected with sensitive strains who are undergoing chemotherapy, especially prolonged therapy. This is most commonly observed in tuberculosis in which the prolonged therapy required, combined with the marked tendency of the microorganism to develop resistance to the kinds of chemotherapeutic agents used - streptomycin, p-aminosalicylic acid (PAS), and isoniazid—facilitates the emergence of resistant strains of the tubercle bacillus. When such infections are treated with a combination of two chemotherapeutic agents, the occurrence of resistant strains is markedly reduced. Many detailed studies have been made, and in a representative investigation85 it was found that when patients were treated with isoniazid alone, 6 per cent yielded isoniazid-resistant strains of tubercle bacilli at the end of one month, and 58 per cent at the end of six months. When the disease was treated with combined antimicrobial substances, streptomycin in combination with PAS or with isoniazid, at the end of three months only 2 per cent of patients yielded streptomycinresistant strains in the first instance and isoniazid-resistant strains in the second. Still other patients treated with PAS only, prior to the institution of combined therapy with streptomycin and PAS, showed streptomycin-resistant strains in 70 per cent at the end of three months of combined therapy when their tubercle bacilli had been initially resistant to PAS, but this was found in only 9 per cent of those whose bacilli were initially sensitive to PAS.

The suppressive effect of combined therapy on the emergence of resistant strains supports the assumption that the resistant strain is made up of the progeny of a resistant mutant, and the growth of a mutant resistant to one of two chemotherapeutic agents used in combination is suppressed by the other. Assuming that the appearance of such mutants is a random, and therefore independent, event, the probability of occurrence of a double mutation is given by the product of the probabilities of each and becomes extremely small.

resistance.27, 110 Un-Infectious drugfortunately the mechanisms of acquiring drug resistance are not always this simple. at least among the gram-negative enteric bacilli. It was found by Japanese workers in 1960 that multiple resistance of dysentery bacilli, involving streptomycin, chloramphenicol, tetracycline, and sulfonamides, is transferred en masse, not only to other shigellas but also to other enteric bacilli, in mixed culture and independent of the sex factor (see below). The transfer of such multiple resistance is a function of an episome⁹⁰ designated the R(resistance)-factor which is independent of the cell chromosome and, in the automonous (i.e., cytoplasmic) state replicates more rapidly than the chromosome. It has been found³ that the transfer of multiple resistance is dependent upon two factors, the R-factor and a separate transfer factor; the latter is functional in the transfer of episomal factors in addition to the R-factor. The earlier designation of the R-factor as the RTF, or resistancetransfer factor, is thus inappropriate. The interrelationship is demonstrable in, for example, an experiment in which three strains of bacteria-one containing the Rfactor alone, another only the transfer factor, and the third neither-are grown in mixed culture. Resistance is transferred to the strain containing only the transfer factor. and as well to that containing neither, but the R-factor would not be transferred in the absence of the transfer factor. Transfer of multiple drug resistance also occurs in vivo in the intestinal tract.⁵³

The possible, or even probable, development of resistant strains of enteric pathogens, such as Salmonella, in domestic animals through the use of feeds containing antibiotics for growth-promoting purposes has been a matter of considerable concern.⁷¹

Induced resistance. Almost as soon as the antibacterial chemotherapeutic agents became available it was found that strains of microorganisms resistant to them could be readily produced in the laboratory. Induced resistance cannot be considered to differ fundamentally from the processes of development of resistant strains isolated in increasing proportions with the general use of antimicrobial drugs. The infected individual undergoing treatment constitutes a selective environment conducive to the survival of resistant mutants, while in induced resistance the selective environment is that provided by the antibiotic-containing culture medium.

The usual procedure is to grow the bacteria in vitro or in vivo in the presence of increasing concentrations of the antimicrobial substance. This may be carried out by serial culture, or by culture on the gradient plate. The latter is prepared by allowing an agar medium containing the antibacterial substance to solidify at an angle in a petri dish and overlaying it with medium lacking the substance. The activity diffuses from the lower to the upper layer, giving a gradient in concentration on the surface of the medium from one side to the other. Bacterial cultures are streaked across it in the direction of the gradient, and, after incubation, the growth at the limiting concentration may be streaked into the higher concentration area, etc. Or, with some antibiotics, a series of agar mediums containing varying concentrations of the activity may be inoculated heavily, and the colonies growing out at various concentrations are resistant. Resistant variants may be produced in vivo by animal passage in the case of pathogenic microorganism in experimental animals treated with subcurative amounts of chemotherapeutic agents.

Resistance may appear relatively rapidly, even in the first subculture, or more slowly over successive transplants. The maximum attainable may be of a low order, a few hundred-fold or less in the maximum non-inhibitory concentration of the activity, or many thousand-fold. The degree of resistance and the rate of its emergence are, as indicated earlier, to a considerable extent characteristic of the microorganism and of the antimicrobial agent. The resistance so produced persists, to a greater or lesser extent, through successive subcultures in the absence of the chemotherapeutic substance.

For example, the development of penicillin resistance usually requires a considerable period of adaptation of the parent strain to increasing concentrations of the antibiotic. A resistance to several thousand-fold the initial inhibitory concentration may be attained with staphylococci, but only of a much lower order, ten-fold to a hundred-fold with streptococci and pneumococci. In contrast, streptomycin resistance appears very rapidly, often on first subculture, in many kinds of bacteria and is of a very high order. In general, the higher the level of resistance produced, the longer it persists in culture in the absence of the antimicrobial substance. Thus penicillin-resistant streptococci revert relatively rapidly to the original sensitive state, but high resistance to streptomycin appears to be practically permanent.

With the development of antiviral substances it has become evident that viruses also may become drug-resistant, or drugdependent (see below).84 The inhibition of picornaviruses in cell culture by guanidine. or by $2-(\alpha-hydroxybenzyl)$ -benzimidazole has been described elsewhere (Chap. Six). These substances are virustatic and viral replication in cell culture does not occur in their presence, but if contact is maintained the virus becomes resistant and/or dependent and grows in the presence of the inhibitor. 107 Thus poliovirus may be rendered guanidine-resistant, and the dependent strains are no longer virulent for the monkey.65 Among the DNA viruses, it has been found that herpesvirus may be made resistant to 5-iodo-2'-deoxyuridine, and infectious bovine rhinotracheitis virus may become resistant and dependent upon the fluoro and bromo deoxyuridines. 100

Dependent strains. The occurrence of variants not only resistant to certain of the antimicrobial substances, but actually requiring them for growth, was observed first

with sulfonamide and Neurospora and streptomycin and meningococcus, and subsequently has been found to occur with some frequency. Such variants are known as dependent strains.

The significance of this kind of variant lies in the fact that it suggests that some antibiotic substances function as specific metabolic inhibitors. There are two more obvious explanations of this phenomenon. One, the metabolic reactions of the microorganism have become so altered that the analogue has displaced the original metabolite in a metabolic sequence, and is now required. An alternative possibility is that dependent microorganism produces antagonist (see below) in such large amounts that it is toxic, and the antimicrobial substance must be present to neutralize this toxicity and allow growth. The sulfonamidep-aminobenzoic acid relation has been discussed elsewhere, and in this connection it is of particular interest that p-aminobenzoic acid acts as an antimetabolite on sulfonamide-dependent variants. A similar phenomenon has been described for the antimetabolite pyrithiamin. It is not known whether certain of the antibiotics, such as streptomycin, function as antimetabolites, but the occurrence of dependent variants suggests that this may be true.

When pathogenic microorganisms are made drug-dependent, they are unable to infect a susceptible animal host unless the animal is concurrently treated with the chemotherapeutic agent. Such drug-dependent strains have been of considerable interest as possible immunizing agents against infectious diseases, such as brucellosis, to which conventional heat-killed vaccines do not produce an effective immunity.

Cross-resistance. A strain of bacteria may be resistant to more than one kind of antimicrobial substance. When the history of the strain is known, as in the case of variants produced in the laboratory, it is clear that this resistance is of two kinds. One is multiple resistance in which a microorganism has been made resistant, one by one, to a number of antimicrobial substances. This is often encountered in bacterial strains, notably staphylococci isolated in resistant form. The other is cross-resistance in which a variant made resistant to one agent is found to have acquired resistance to another. This is almost uniformly true among

the tetracyclines. Cross-resistance occurs only rarely between unrelated compounds and is of a low order. In relatively rare instances increased resistance to one agent may be associated with increased sensitivity to another.

Physiology of resistance.⁷² Since the antimicrobial substances having therapeutic utility act by inhibition of growth processes, the basis of resistance to such substances is probably to be found in alteration of the metabolic processes of the microbial cell. The resistant variant may be presumed to differ in this respect from the parent strain, and the more obvious possibilities of the way in which this may occur are:

Synthesis of antagonists. The resistant variant may neutralize the activity of the chemotherapeutic agent by production of antagonists in greater than normal amount. This is clear-cut in the case of some sulfonamide-resistant variants. For example, many of the sulfonamide-resistant gonococcus and staphylococcus variants are resistant because they produce excessive amounts of inhibitor, often but not necessarily p-aminobenzoic acid. Similarly, diphtheria bacilli made resistant to the inhibitory effect of the analogue pantolyl taurine synthesize pantothenic acid sufficient to support growth in the presence of the inhibitor.

It is doubtful how far this approach may be generalized. For example, all sulfon-amide-resistant gonococci and staphylococci do not form increased amounts of sulfon-amide antagonist, and some other mechanism is operative. In addition as yet specific antagonists of many antimicrobial substances, especially the antibiotics, are not known.

Decomposition of the inhibitor. Resistance to penicillin has been found to be associated in many, but not all, instances with the production by the resistant bacterial strain of an enzyme, or enzymes, which decompose the antibiotic. The best known of these, and the one apparently responsible for in vivo resistance, is penicillinase, or penicillin β -lactamase, which splits the penicillin molecule containing the lactam linkage to give inactive penicillinoic acid. It is an adaptive, i.e., inducible, enzyme which occurs extracellularly in gram-positive bacteria and is formed by a number of kinds of bacteria, notably staphy-

lococci and Bacillus species. Penicillinase production probably accounts for resistance in almost all strains of penicillin-resistant staphylococci isolated from infections, and it has been shown³⁰ that its induction occurs in vivo as well as in vitro. The penicillinases produced by gram-negative bacteria tend to be set apart, in that they are intracellular and noninducible and are produced in relatively small amount.95 It has been suggested that, since the high lipid content of the cell wall of the gram-negative bacteria makes them relatively impermeable to penicillin, small amounts of intracellular penicillinase suffice to inactivate penicillin in resistant organisms. Some penicillins differ sufficiently in structure, e.g., dimethoxyphenyl penicillin (methicillin) and 3-phenyl-5methyl-4-isoxazolyl penicillin, that they are not decomposed by the β -lactamase, and are valuable in the treatment of infecwith penicillin-resistant bacteria. staphylococci in particular.

Penicillins are inactivated by other enzymes of bacterial origin. Penicillin acylase (penicillin amidase)⁴⁰ splits the peptide linkage by which the side chain is attached to the nucleus of 6-amino penicellanic acid, and the remaining nucleus is relatively inactive. This enzyme apparently plays little or no part in penicillin resistance *in vivo* in part because of its high pH optimum, but it has been extremely valuable in the preparation of "semisynthetic" penicillins (Chap. Six). It is produced by both gram-positive and gram-negative bacteria.²⁵

In some instances, especially in penicillin-resistant staphylococci, decomposition of the antimicrobial agent seems not to be a common mechanism of resistance. It has been reported, 5 however, that multiple resistance of R-factor-containing enteric bacilli is associated with the formation of enzymes that decompose the antibiotics. Intracellular, noninducible enzymes which degrate chloramphenicol, dihydrostreptomycin, and kanamycin have been found in resistant strains containing the R-factor, but not in strains made resistant by adaptation.

Alternative metabolic pathways. A third way in which the variant may become resistant to an antimicrobial substance is by the development of an alternative equivalent metabolic pathway to replace that affected by the agent. The precise nature of the in-

hibiting effect is not known in most cases, but if such an alteration occurs, it might be expected to be accompanied by demonstrable physiological differences between the sensitive microorganism and its resistant variant. There is a great deal of evidence that resistance is accompanied by a variety of changes in metabolic processes such as altered nutritive requirements and impaired carbon metabolism.

For example, the assimilation of glutamic acid by staphylococcus described elsewhere (Chap. Three) in connection with the osmotic barrier, does not occur in penicillinresistant variants, and free glutamic acid is not found in the cell, indicating that the variant has dispensed with the assimilatory mechanism and synthesizes its own glutamic acid. Consistent with this is the general observation that penicillin-resistant staphylococci which do not produce penicillinase no longer require as many preformed amino acids and bacterial vitamins. 15 Among the enteric bacilli, the converse has been observed, i.e., variants showing more complex nutritive requirements may be less sensitive to the antibacterial activity of penicillin than the parent strain.79

More specific changes have been found by a number of workers, especially Sevag and his co-workers.87,93 It has been shown, for instance, that pneumococci resistant to various chemotherapeutic substances have altered flavoprotein enzyme activity, possibly arising from a modification of the protein moiety, and reduced dehydrogenase activity and ability to oxidize glucose and intermediates such as lactate and glycerol. Similarly, streptomycin-resistant coliform bacteria partially or completely lose the ability to metabolize pyruvate anaerobically, and some variants partially replace the normal anaerobic phosphoroclastic metabolism of pyruvate, which is sensitive to streptomycin, with a streptomycin-resistant dismutative process. Pyruvate metabolism is altered also in chloramphenicol-resistant staphylococci so that it is not utilized but acts as a hydrogen acceptor to accumulate additional lactic acid in the medium.⁷⁷ Isoniazid resistance of tubercle bacilli is accompanied by a loss of the ability to produce catalase and a decreased virulence for the guinea pig, and the former may well be related to the observation that hemin antagonizes the antibacterial effect of isoniazid on tubercle bacilli.⁵⁸ Similarly, indophenol oxidase-containing extracts of colon bacilli resistant to chlortetracycline have been found not to be inhibited by the antibiotic, while such extracts of sensitive bacilli are inhibited.⁸⁹

While data such as these make it clear that the physiological processes of many bacteria are appreciably, or even profoundly, altered in resistant variants, available information is as yet too fragmentary to make it possible to discern a pattern in the physiological changes accompanying resistance.

It is not necessary to assume that a single mechanism is operative in the development of resistance to chemotherapeutic agents in that the foregoing are not necessarily mutually exclusive. In fact, the utilization of more than one mechanism in an apparently single adaptation is indicated in the observation that, in the adaptation of coliform bacilli to sulfanilamide there is first a reduction in the prolonged lag period interpreted as the development of an alternative metabolic sequence, followed by an enhanced growth rate attributable to the formation of an antagonist.

ADAPTIVE ENZYMES^{23, 80, 81, 97, 99}

The ability of a microorganism to decompose a substrate may be very rapidly enhanced in the presence of that substrate. Although known for many years, this particular kind of bacterial variation has been the subject of renewed interest as enzyme adaptation. The enzymes which may be formed under the influence of the presence of substrate have been called adaptive enzymes in contrast with those which are formed whether or not the substrate is present, the constitutive enzymes. The formation of adaptive enzymes may be demonstrated by cultivation of the bacteria in a medium containing the substrate and then testing the organisms for enzymatic activity, or a washed suspension of bacteria grown in the absence of the substrate may be mixed and incubated with it. In the first instance, activity is immediately apparent, the bacteria having formed the adaptive enzyme during growth, and in the second a latent period varying from 30 minutes to two to

four hours occurs before rapid decomposition of the substrate begins.

A number of bacterial enzymes are adaptive in that they are formed wholly or in large part in the presence of the substrate. Among these, the β -galactosidase of the colon bacilli and penicillinase formation by B. cereus have been widely used as model systems. The amino acid decarboxylases are formed for the most part in the presence of the substrates. Many of the bacterial proteases are adaptive enzymes, for only small amounts of the enzymes are formed in the absence of protein, but filtrates from cultures in protein-containing mediums are actively proteolytic. Various other bacterial enzymes are adaptive also; thus, the enzyme tetrathionase, responsible for the reduction of tetrathionate to thiosulfate by Salmonella, and nitratase, which catalyzes the reduction of nitrate to nitrite by the colon bacillus, are adaptive as is the enzyme penicillinase formed by Bacillus species. The distinction between adaptive and constitutive enzymes may not always be a sharp one, for frequently, if not invariably, as in the case of the bacterial proteases. the adaptive enzyme is not completely lacking in the absence of the substrate.

The property of inducing adaptive enzyme formation is not necessarily confined to the substrate. Thus in the β -galactosidase adaptation of the colon bacilli, melibiose and galactose are inducers but not substrates, neolactose is a substrate but not an inducer, and thiophenyl-β-galactoside is a competitive inhibitor of the enzyme activity but is not an inducer. Lactose is, of course, both substrate and inducer in this instance. The adaptation, as assayed by the ability to ferment lactose, is more complex than this, for it is now clear that the ability to ferment involves not only the β -galactosidase, but the cell must also be permeable to lactose. This last is a function of the enzymatic activity designated permease^{20, 22} which is also an adaptive enzyme (Chap. Three).

The question of whether or not the formation of an adaptive enzyme is associated with bacterial multiplication is one of considerable interest. In general it appears that some degree of multiplication is essential (cell division occurs even in suspensions of washed bacteria), and it has been shown in the formation of β -galacto-

sidase by colon bacilli that the amount of enzyme was proportional to the number of new cells. In the majority of instances actual proliferation of the cells need not occur, though synthesis of the enzyme takes place during the latent period. The adaptive formation of formic hydrogenlyase by nonproliferating coliform bacilli requires a source of both energy and amino acids and is inhibited by assimilatory poisons such as nitrate and nitrite60 and by x-irradiation of the cells. In general, adaptive enzymes are formed under conditions of nitrogen starvation when there is no net increase in the number of cells, and this is probably a consequence of protein turnover in the nonproliferating cells.

It is both relevant and significant to note that substantially all, rather than a select few, of the cells subjected to the inducersubstrate participate in the formation of the enzyme. This, and the requirement for amino acids, indicates total synthesis of the enzyme. The observed lag in the appearance of enzymatic activity is presumably due to activation of the enzyme, for total synthesis is very rapid; penicillinase, for example, is formed within 30 seconds. Adaptive enzyme formation is inhibited by growthinhibiting antimicrobial substances this is taken to suggest that these substances inhibit synthesis of constituent protein by the bacterial cell. It is of interest in this connection that kinetic considerations have shown that at least in the adaptive formation of penicillinase by Bacillus cereus, the inducer-substrate, penicillin, cannot be incorporated into the enzyme but must function catalytically, in that each mol of penicillin fixed or destroyed gives at least 10 mols of the enzyme.80

The phenomenon of enzyme adaptation has been made use of, especially by Stanier, in the elucidation of adaptively controlled metabolic pathways. In a chain reaction controlled by adaptive enzymes represented

$$A \xrightarrow{\sim} B \xrightarrow{\sim} C \xrightarrow{\sim} D \xrightarrow{\sim} etc.$$
 $E_a \xrightarrow{} E_b \xrightarrow{} E_c \xrightarrow{} E_d$

the reaction A B is catalyzed by the adaptive enzyme E_a, B-C by enzyme E_b, etc. When any intermediate is supplied, the chain reaction continues to completion since the product of one reaction is the substrate for the next, and in its presence the appropriate adaptive enzyme is formed. Thus substances presumed to be intermediates can be tested for actual participation in the chain reaction; *i.e.*, if the compound is dissimilated immediately by cells adapted to substrate A, for example, but not by cells not adapted to A, it is highly probable that it is an intermediate. This technique has been applied, in whole or in part, to analysis of adaptive patterns in the oxidation of Krebs cycle components, aromatic compounds including tryptophan, tyrosine, and purines.

CONJUGATION AND RECOMBINATION^{2, 39, 61}

The possible occurrence of sexuality among bacteria and related microorganisms has permeated the interpretations of variation phenomena for many years, but it was not until 1947 that unequivocal evidence for the recombination of marker characteristics, and inferentially conjugation, between unlike cells was presented by Lederberg. In essence this consisted of the isolation of radiation-induced variants of the parent K-12 strain of coliform bacilli, characterized by physiological differences such as requirements for specific amino acids. When such variants are grown in mixed culture, progeny may be isolated in which the markers occur in the possible combinations, including combinations not present in the original strains, and in kind and proportion to be expected on the assumption of a conjugative recombination.

Bacterial sexuality.⁵¹ It would appear to be implicit in the occurrence of recombination that the cells which conjugate differ from one another, *i.e.*, that conjugation has a sexual basis. It is now clear that there is a sex factor, F, present in some cells and not in others. These are designated F+ and F-, the F+ cells being donors, or males, and the F- cells the recipients, or females. In the F+ cell the F factor, like the R factor described above, is autonomous and replicates independently of the bacterial chromosome, though at about the same rate, and there is one, or possibly as many as three, per F+ cell.

When F+ cells are mixed with an excess of F- cells, recombination is a relatively rare event; about one recombination occurs per 10,000 F+ cells. The observed recom-

36

bination is due to the presence of a mutant of F+ cells, designated Hfr (high frequency recombination), in which the F factor is integrated in the bacterial chromosome. Thus when conjugation occurs between an Hfr cell and an F- cell, all or part of the bacterial chromosome is transferred rather than only the F factor.

Recombination. In the mechanics of conjugation a bridge, or tube, is formed between the reacting cells, and the chromosome, including the sex factor, is transferred linearly. The entire process is complete in 90 to 100 minutes. If it is interrupted, as by agitation of the bacterial suspension in a blender, transfer is only partial, and even without agitation transfer is usually incomplete. When chromosome transfer is effected, the markers are transferred in precisely the same linear sequence from all mating cells, the origin of the sequence being the point at which the F factor is integrated with the chromosome. When transfer is incomplete, the frequency of transmission of markers is a function of their distance from the point of origin, and the sex factor is transferred last. Thus the bacterial chromosome may be mapped as a linear sequence of genes. The transferred markers are the result of recombination between the Hfr chromosome, or chromosome fragment, and the chromosome of the F- recipient cell. The information contained in the individual gene, or genetic element (see below), so transferred is a function of the sequence of purine and pyrimidine bases making up the nucleic acid chain. The coding of genetic information in this way has been described earlier (Chap. Five).

A number of terms have been coined for definition of the various spatial relationships. A recon is a unit of nucleic acid not subdivisible in recombination, and consists of about 12 nucleotide pairs. A muton is the shortest length of the nucleotide chain which must be altered to produce a mutant, a maximum of about five nucleotide pairs. Finally, a cistron is the unit of length, about 4000 nucleotide pairs, required to produce some kind of integrated function, but function can be impaired by mutations at many different locations on a cistron.

As has been pointed out earlier (Chap. Three), it is highly probable that the bacterial chromosome, at least in K-12 coliform

variants, is circular, and its linear transfer during conjugation involves breakage. In Hfr cells, breakage appears to occur at the point at which the F factor is inserted into the chromosome. It would seem necessary, too, that the Hfr chromosome be in contact with the outer portions of the bacterial cell to be available for transfer to the recipient cell. Such contact is with mesosomes, invaginations of the cytoplasmic membrane, and the point of contact is the locus of the sex factor. Conjugation probably involves appropriate receptor sites, or their equivalent, and the inhibition of mating by periodate through temporary destruction of the virility of F+ cells suggests that some surface polysaccharide-containing substance is involved.96 Presumably contact with the recipient cell initiates formation of the connecting tube, and triggers the transfer of the broken chromosome. The actual implementation of the transfer is not clear; it has been suggested that chromosome replication occurs concurrently with transfer, the force of replication providing for the transfer, and also that replication occurs prior to transfer but before the linear chromosome assumes circular form.

The separation of the F factor from an integrated position in the chromosome to assume autonomous character as in the F+cell is considered to be a result of crossing over. This is supported by the occurrence of variant autonomous F factors which include genetic markers that are adjacent to it when it is integrated with the chromosome. Male cells containing such variant F factors are known as F' cells and are able to transfer such associated markers to F-cells to give recombinants.

While the foregoing has been worked out substantially in its entirety by study of variants of the K-12 strain of E. coli, there is sufficient homology with certain other enteric bacilli to allow recombination across "species" lines. Recombination between Escherichia and Shigella and between Escherichia and Salmonella117 is now well established. Utilization of the former has been particularly informative with respect to the virulence of Sh. flexneri and has made possible the development of appropriate hybrids as immunizing agents (Chap. Twenty-two). At the same time, it is by no means certain the extent to which such findings can be extrapolated to other kinds of bacteria.

Viral recombination.²⁶ It is of particular interest that recombination occurs between variant or closely related strains of viruses, in that these agents appear to be genetic mechanisms uncomplicated by an associated physiological system.

Recombination between viruses occurs when sufficiently closely related strains or variants are "grown" together as mixed infections of the same host cell. As pointed out elsewhere (Chap. Four), mixed infection rather than interference is produced under appropriate experimental conditions. The progeny resulting from such mixed infections are quite analogous to those produced by bacterial recombination, i.e., types appear that are combinations as well as replications of the strains making up the inoculum. The observation that lysogenicity, i.e., apparently latent infection of the bacterial cell with temperate strains of bacterial virus, also behaves as a stable genetic character in recombination experiments with K-12 and B coliform bacilli and the λ coliphage serves to link the phenomenon of recombination with that of transduction (see below).

There seems to be no morphological aspect of viral recombination in that the mature virus particle does not exist during active multiplication within the host cell, and conjugation of vegetative virus resolves to an interaction between two kinds of viral nucleic acid. The nature of the exchange of hereditary elements is best understood for certain of the bacterial viruses, using markers. such as minute plaque formation, ultraviolet sensitivity, host specificity, and rapidity of lysis. Among these viruses the "mating" that gives rise to recombinants is, in effect, multiple in that the primary redistribution of genetic material is followed by other matings between recombinants and thus resembles recombination between populations rather than individuals. The occurrence of such successive matings is indicated by the appearance of progeny which could not have been formed by any kind of exchange between the two parental viruses.14

Analogous recombination occurs among several of the animal viruses.^{55, 83} The markers are characters such as pathogenicity for the mouse lung and the embryonated hen's egg, neurotropism, plaque morphology,¹⁰⁶ and antigenicity. The results of recombination experiments have shown, not only that recombinants occur, but that there

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are linkage groups, as for example between mouse lung pathogenicity and antigenic character, and, further, that noninfectious virus inactivated by ultraviolet irradiation may enter into combination with infective virus. This last is suggestive of the transformation phenomenon (see below), possibly implying that the latter may be a special case of the recombination phenomenon. Recombination with the production of hybrids also occurs among the poxviruses and between antigenic types of poliovirus.³¹

TRANSDUCTION (TRANSFECTION)^{21, 62, 66}

The method of transfer of genetic material from one cell to another based on a sexual system and involving recombination of entire blocks of genes in intact cells is the conventional one in that it is shared by all kinds of living organisms, including microorganisms. In addition to recombination, there occurs among the microorganisms a transfer of genetic fragments, mediated by bacterial viruses, or introduced into the cell directly as macromolecules of polymerized nucleic acid. In either case the process is one of transduction of genetic elements, but the term transduction has been applied to the phage-mediated transfer, while the direct application of nucleic acid has been called transformation, because it was first observed in the transformation of immunological types.

In phage-mediated transduction the genetic material is transferred from the donor cell to the recipient by bacteriophage. Virulent phage may act as a transducing agent under appropriate conditions, but most often the transduction is by temperate phage. The lysogenic donor cells containing the marker are broken down by induced phage lysis, recipient cells are infected, and the marker is present in those which survive by becoming lysogenic. This is studied in the most part as transduction between strains of a given bacterial species, or different species, but it also occurs, like conjugation, across generic lines, between coliform bacilli and dysentery bacilli.68

Two kinds of transduction are to be distinguished. The one, sometimes called gen-

eralized transduction, is casual in the sense that the fragment of donor cell genetic apparatus carrying the marker is injected into the recipient together with viral DNA, but is not a part of it. About three per million phage particles are transducing for the marker, or about one transducing particle for every 300 to 500 bacterial donor cells lysed, so that relatively few of the lysogenic recipients are found to contain the marker. It is characteristic of transduction that usually only a single marker is transduced because the transducing particle contains only a genetic fragment from the donor cell, but occasionally markers may be so closely linked that both occur in the same fragment.

The markers may be of various kinds. Variants may be isolated which are characterized by markers such as specific nutritive requirements, fermentative abilities, resistance to chemotherapeutic substances, or antigenicity. Lysis of the lysogenic variant is induced, as by treatment with ultraviolet light, and the marker characterizing the variant is transferred together with lysogenicity, to a susceptible strain of bacteria. In this way a drug-sensitive strain of bacteria may be made drug-resistant, or, by transduction of antigenicity, new serotypes may be created.

The essentially passive role of the bacterial virus is substantiated by the ability of ultraviolet-irradiated phage to transduce, e.g., infectivity is destroyed by radiation more rapidly than the transducing factor, and transduction may be accomplished without inducing lysogenicity in the recipient. The virus thus functions as only a carrier of genetic fragments resulting from the lysis of the donor cells, and as a means by which such fragments are introduced into the recipient cell. The fragment suffices to replace the homologous gene in the recipient, the exchange occurring quite soon after the fragment enters the cell, and the prior homologue does not reappear. Such homologous replacement, as for example of flagellar antigens of Salmonella, suggests an organized genetic system in the recipient bacterium but offers no clue as to how the homologues are associated prior to the elimination of one or the other.

The other kind of phage-mediated transduction derives from the intimate relation between prophage and the host bacterium in the lysogenic state. Although autonomous as an infectious particle, the temperate prophage becomes a part of the bacterial genetic apparatus, and the induction of lysogeny is, in effect, a transduction. In recombination experiments the prophage segregates as if it were a bacterial marker and, further, it may be transduced as prophage by another bacterial virus. The λ coliphage is linked with galactose fermentation, suggesting that in this case the phage may occupy a predetermined site in the bacterial genetic apparatus. The fact that lysogenicity may be multiple, to as many as three different prophages, shows, however, that more than one site may be occupied by prophage. It has been suggested that the genic factors so imposed on the genetic apparatus of the host cell are not always a part of that apparatus and may be regarded as episomes.⁵⁰

Changes correlated with lysogenic conversion differ from those of generalized transduction in that they are present in all, rather than a small portion, of the lysogenic progeny and cannot be dissociated from the presence of prophage. The most interesting of these is the toxigenicity of lysogenic diphtheria bacilli.

It appears to be definitely established that all toxigenic diphtheria bacilli are lysogenic, though all lysogenic strains are not necessarily toxigenic, and that the induction of toxicity is inseparable from phage. This phenomenon has been studied in detail^{10, 37, 38} and may be described briefly. When any strain of diphtheroid bacilli sensitive to phage β is made lysogenic, it invariably becomes toxigenic; when lysogenicity is lost toxigenicity disappears but may be induced again when the strain is again made lysogenic. Another strain of phage, y, differing in host range, produces lysogeny of sensitive strains of bacteria, but these are not toxigenic. Recombinants of β and γ prophages have been isolated, of which one, β' , has the host range of β but does not produce toxigenicity, while another, γ' , has the host range of γ and does produce toxigenicity. Further, bacterial strains doubly lysogenic for β and γ are toxigenic. Thus the prophages behave as if they were bacterial genes, with toxigenicity dominant, affecting the metabolic processes of the bacterium concerned with the production of the iron-deficient porphyrin complex that is diphtheria toxin.

TRANSFORMATION29, 98

Transformation is a transduction accomplished by direct treatment of the recipient cell with nucleic acid from the donor cell and thus relates to viral replication via infectious nucleic acid (Chap. Four). It was first observed with pneumococcus in experiments in which the immunological type, determined by the antigenicity of the polysaccharide capsular substance, was changed or transformed. It also occurs with respect to characters other than antigenicity on the one hand, but, in addition, is to distinguished from other kinds of immunological changes, such as those associated with the S-R dissociation, environmental modification of antigenicity such as the temporary repression of flagella and flagellar antigen formation in the presence of dilute phenol.

It was suggested by Theobald Smith and Reagh in 1904 that bacteria might be immunologically altered by residence in different hosts, although they were unable to demonstrate such changes, and much later, Holtman^{46, 47} showed that enteric bacilli could acquire the antigenic specificity of Forssman antigen by growth in the presence of this substance, the specificity persisting through 20 to 50 transplants of the modified bacilli. The latter probably accounts for the irregular occurrence of Forssman antigen in a variety of bacteria and the presence of Forssman antibody in presumably normal animals, such as rabbits, in which the antigen does not occur, as a consequence of infection with such bacteria.

The variation now known as transformation was described in 1928 by Griffith, who inoculated animals with avirulent R strains of pneumococci originally derived from, for example, type 1 pneumococcus, but no longer having type specificity, together with a killed vaccine of encapsulated, typespecific pneumococcus of another type, such as type 2. Following development of an infection, living virulent pneumococcus of the vaccine type, in this example type 2, were found, indicating that the R variant from type 1 had been converted, or transformed, to the S virulent encapsulated type 2, i.e., had acquired the ability to synthesize the type-specific capsular polysaccharide of the vaccine strain. This phenomenon was subsequently studied in detail by others,

especially Avery and his co-workers, and could be made to occur in vitro.

The transformation reaction. Transformation in vitro occurs in several distinct stages. There is first a preliminary period of growth in an appropriate medium (see below); second, a period during which transformable cells make their appearance, usually during the latter part of the logarithmic growth phase; third, a short period, perhaps 10 minutes, during which the transforming principle is taken up by the recipient cells; fourth, a longer period, 45 to 60 minutes, during which phenotypic expression of the transformation develops; and, last, the appearance of progeny of the transformed cells which show the acquired character and breed true indefinitely.

Transformation factors. There are three variables in this process. The first is the substance originating in the donor cells and transmitted to the recipient, the transforming principle or TP. This was first isolated from pneumococcus, and subsequently from other bacteria, as a highly polymerized deoxyribonucleic acid; depolymerization or small changes such as a minor amount of deamination result in inactivation. The material retains its activity during purification and fractionation, as in resin columns, and appears to have a molecular weight of about 700,000, though there is reason to believe that only a portion of the macromolecule is active. Only a fraction of the TP is irreversibly adsorbed by the treated recipient cells, and most of it can be washed off or decomposed by deoxyribonuclease without interfering with the transformation. Kinetic studies indicate that one molecule per recipient cell is sufficient to transform, but when TP is present in concentrations greater than 10 molecules per cell, the rate of transformation is less than the extrapolated rate, suggesting some kind of interference.

The second variable is known as the serum factor which is, of course, present in *in vivo* transformation, but which must be supplied in the medium when transformation occurs *in vitro*. This factor is differentiable by dialysis into two fractions. The dialyzable fraction is replaced by pyrophosphate, and the nondialyzable fraction by bovine serum albumin. The manner in which these factors function is unknown. In some kinds of transformation a third component of the serum factor, agglutinating antibody to the bacteria,

is required, but this is a special case in that it is required only in certain immunological transformations.

The third variable is the susceptibility of the recipient cells to the action of the transforming principle. As implied above, all the cells in a bacterial culture are not capable of accepting the transferring principle. Cells which are, are said to be competent, and competent cells appear later in the logarithmic growth of the usual culture. The proportion is not high, ranging from one to ten per thousand cells. They occur in peaks, and under some conditions these peaks occur periodically. The peak persists in time only 15 to 20 minutes and is apparently associated with maturation of the individual cell; the peaks and their periodicity are a result of a partial synchronization of cell division. Cell division may be approximately synchronized17 (Chap. Four), with increase in the proportion of competent cells to as much as 17 per cent. That competence is associated with a physiological state is indicated by the occurrence of transformation when TP is not present in the growth medium but on transfer of competent cells to a medium containing TP.50 Further, competent cells may be frozen and still retain competence for as long as six weeks, though viability may persist for 18 months when the cells are stored at -20° C.

Other transformations. Transformation is not limited to that of pneumococcal types but has been demonstrated for immunological character in Hemophilus, meningococcus, coliform bacilli, and Shigella. Characters other than antigenicity are transformable also, including such diverse properties as fermentative abilities and drug resistance. In fact, it may be supposed that almost any bacterial characteristic may be reproduced by transformation. Transformation may also occur across species, or even genera if the pneumococcus is to be regarded as so separated from the streptococci, for streptomycin resistance has been exchanged between these microorganisms, in both directions via soluble DNA.

Fibroma-myxoma.⁵⁶ A closely similar, if not basically identical, phenomenon was described in 1936 by Berry and Dedrick, and is known as the Berry-Dedrick phenomenon or as the fibroma-myxoma virus transformation. The fibroma and myxoma viruses are tumor viruses infecting the rabbit, and be-

MUTATION 243

long to the pox group (Chap. Thirty-seven). The former produces a benign, localized tumor which begins to regress within a few days, while the latter produces a fulminating rapidly fatal infection. The transformation was originally described in substantially the way Dawson described type transformation of pneumococcus in vivo. Myxoma virus may also be transformed into fibroma virus in tissue culture. The transforming principle is effective not only as a heat-inactivated, DNAse-resistant preparation of fibroma virus, which electron micrographs indicate

to be vegetative virus, but also as a ureasolubilized preparation which is sensitive to DNAse.

There is evidence that this phenomenon may be a general one, at least among the poxviruses, for Japanese workers have reported similar transformations in tissue culture of myxoma virus into vaccinia virus and ectromelia (mousepox) virus, and of ectromelia virus into vaccinia virus. ⁴¹ In a general sense such transformations involve the possible integration of viral nucleic acid in the genome of animal cells.⁵⁹

The Nature of Microbial Variations

It is clear from any consideration of microbial variation that all, or nearly all, of the characteristics made use of in the differentiation and identification of these forms are subject to alteration. While many of them do not yet have counterparts in classic genetics built around differentiated higher organisms, there is no reason to believe that other than substantially the same mechanisms are operative for all living things.

At the same time, studies of microbial variation have broadened tremendously concepts of genetic mechanisms, and it has become clear that variability need not be the random process of selection of the fittest among spontaneously arising genic mutants, but may, in fact, be induced in the broad sense. The facility with which inductive or seemingly inductive changes may occur is possibly related to the more intimate association of the germ cell of the microorganism with environmental influences as contrasted with the protection of the barrier of somatic cells intervening between the environment and germ cells of higher forms.

Further, the concept of virus as an independent gene or group of genes, tentatively suggested much earlier, tends to be substantiated with the demonstration of situations, such as lysogeny, in which it becomes difficult to formulate definitions of virus and gene which are mutually exclusive. It is equally significant that parasitism is here extended to a genic level with extinction of the autonomy of the two agents.

It may be taken as fully established that the differential characters of microorganisms are the phenotypic expression of the func-

tioning of a genetic apparatus whose component parts behave as discrete entities, which may or may not be linked, and thus have the attributes of genes of classic genetics. It is equally well established that many differential characters depend, at least for their expression, on the presence of a specific substrate or other inducer in the environment. The question at hand is that of determining which of these mechanisms, or modifications of them, are altered with the appearance of microbial variants, and their relative importance in the phenomena of microbial variation. In general, there is a tendency to subordinate one or the other, and to take the view either that genic mutation is relatively all-important or that the great majority of microbial variants represent an adaptation in the sense of a response to environmental induction.

MUTATION13, 49

It is already apparent from foregoing considerations that genic changes may be induced in microorganisms in the phenomenon of transduction, either phage-mediated or direct as in transformation. The definition of mutation must inevitably be broadened from its classic conventional meaning to include these kinds of induced genic modification. So it is not a matter of whether genic changes occur, but rather one of, first, whether spontaneous undirected mutations occur and, second, the extent to which microbial variants represent a result of this process.

Spontaneous mutation. Randomness is

the identifying characteristic of spontaneous mutation in that the mutation occurs as a result of the operation of an indefinitely large number of small independent causes rather than one, or at the most a few large and important causes. The necessity for studying microorganisms as populations requires that a spontaneous mutant be selected so that it may become predominant and characterize the population in order to be detectable. It is an unfortunate consequence of this that a selective environment, whose selectivity is dependent upon the presence of a substrate or other potential inducer, is interposed at one point or another. The occurrence of spontaneous mutation must be inferred from population behavior* and cannot be of an unequivocal nature, but when such evidence is derived in different ways, its cumulative weight becomes great.

Many bacterial variations behave as if they were a consequence of spontaneous mutation, and the mutants selected, rather than induced, by the culture medium. For instance, the frequency with which variants appear is of the same order as the mutation rates established in higher forms, e.g., one variant cell per 10^6 to 10^9 parent cells. This rate may be increased by treatment with radiation or other mutagenic agents such as nitrogen mustards (β -chloroethylamines), though not with colchicine. These variants breed true, in fact could not be found if they did not, and behave in recombination experiments as genic entities.

If such variants occur spontaneously, it follows that their number is directly related to the size of the population, and in small populations would fluctuate widely. This consideration is the basis of the fluctuation test in which a parent population sufficiently large to contain some mutant cells is divided into many small parts which are grown independently. If only an occasional mutant is present in the parent population not exposed to the selective environment, its appearance in subcultures in the selective medium should fluctuate erratically rather than appear uniformly. Many microbial variants do show such fluctuation. The criticism that the fluctuation test shows only randomness, not necessarily spontaneous mutation, is, of course, a legitimate one.

A more direct method of demonstrating the occurrence of mutation in the absence of possible inducing substrates, i.e., preexistence of the mutant relative to its contact with the selective environment, is the replicate plating method. A plate culture on a nonselective medium is allowed to grow for a short time to produce microcolonies. The growth is then impressed on the surface of sterile velveteen, and the pattern of growth is imprinted on plates of selective mediums. Following incubation the growth patterns and the colonies on a selective medium, as for example one containing an antimicrobial substance, are compared, and if the colonial growth on the selective medium corresponds to that on the nonselective medium, it is inferred that the variant clone was pre-existing with respect to the selective agent.

Furthermore, the occurrence of conjugation among the microorganisms with consequent recombination of markers shows that such markers, gene-controlled enzyme systems, 69, 86, 115 segregate in a manner completely consistent with the occurrence of their determinants in a linear arrangement. Too, as indicated earlier, the process of transfer of genic material during conjugation may be interrupted to show a sequence in the markers transmitted. In consequence, it has been possible to prepare maps of gene loci for bacterial viruses as well as bacteria, but the necessary detailed information is not as yet available for the animal viruses.

The cumulative weight of this kind of evidence is considerable, and is regarded by many as establishing beyond reasonable doubt the selection of spontaneous mutants as the basis of the great bulk of microbial variations.

Its application to the phenomenon of drug resistance may be taken as a representative example. Suppose Bacillus X is adapted to grow in the presence of an antimicrobial agent in concentrations which inhibit the growth of the parent strain. The nature of the resistance, e.g., the development of alternate metabolic pathways, decomposition of the drug, etc., is, of course, irrelevant here. The process of adaptation is conceived as the spontaneous appearance in the parent population of resistant mutants which grow in the concentration of the drug applied while the parent strain does not. Resistance may be increased, as described earlier, by

^{*}For a detailed discussion of the relevant statistical theory see references 6 and 7.

cultivation of the variant in successively higher concentrations, and in each of these new mutants appear spontaneously which are characterized by an even greater drug resistance until some maximum is reached. This is the stepwise rather than uniform rate of emergence of resistance which is regarded as additional evidence of the spontaneous, rather than induced, appearance of resistant mutants.

On continued culture in the absence of the drug, resistance disappears gradually at about the same rate that it was acquired, and this is interpreted as a consequence of a series of back mutations to sensitivity to the drug.

INDUCED ADAPTATION

The wide range over which microbial adaptation occurs, and the specificity of the adaptations, has suggested to many workers that the adaptive process is a directed one rather than a chance occurrence, that is to say, is induced by the chemical character of the environment, rather than a consequence of the selection by the environment of spontaneously occurring variants. Further, the usual dependence upon the presence of the substance toward which the adaptation is directed suggests that the relation between the two is causal rather than coincidental. There is no question that specific induction occurs in the phenomenon of enzyme adaptation described earlier, and it is established that the synthesis of enzyme is specifically induced, at least in the sense of being markedly stimulated, in the presence of the substrate. The theoretical aspects of this phenomenon, upon which any generalization is dependent, have been examined by Yudkin¹¹⁶ who has formulated the concentration relationships of enzyme and substrate in mass action form. This approach has been further elaborated by Mandelstam,70 and shown to fit closely with the observed behavior of these systems.

The physiological processes of the metabolizing cell may be regarded as a series of interrelated reactions in which the product of one is the substrate of another, as in the catalysis of respiration. Or the linkage may be cyclical, one of the later reactions in the sequence providing an intermediate functioning in one of the earlier reactions in

conjunction with some other substrate, as in the cyclical processes of carbohydrate metabolism such as the alcoholic fermentation and the Krebs cycle. The individual reactions are determined by the concentration of intermediates, the amount of enzyme, and the reaction velocities.

Enzyme balance. If the kinds and proportions of enzymes present within the cell are affected by the nature and concentration of substrates present, adaptation can consist of an adjustment of the cellular physiology as a whole to utilization of the substrates with maximum efficiency. This is, in effect, a generalization of the phenomenon of enzyme adaptation and has been most fully developed by Hinshelwood.45 From this point of view, the sequence of metabolic events can be visualized as follows. A steady state occurs during logarithmic growth, but with the exhaustion of food materials, accumulation of metabolic products and the like, there is cessation of the maximum growth rate, concentrations of diffusible intermediates fall, and the individual enzymes decay and alter in their relative proportions as the system moves toward a new equilibrium. On transfer to fresh medium, readjustment is initiated during the lag period with the building up of concentrations of intermediates and a synthesis of enzymes in a shift toward an equilibrium consistent with maximum growth rate. Thus, the relative proportions of the elements of the catalytic system and the concentrations of substrates of the individual reactions exist in a series of metastable states of enzyme balance under ordinary culture conditions.

There is a great deal of evidence that alterations of this kind occur. For example, the formation of bacterial deaminases is suppressed during rapid growth in the presence of fermentable carbohydrate and does not occur at acid reactions but decarboxylases are formed at an acid reaction, and both kinds of enzymes are formed in the later stages of culture growth; the kind and proportion of enzymes present are a function of the age and condition of the culture. Similarly, the products of carbohydrate metabolism depend upon environmental factors, again a matter of balance in the function of the cellular enzymes.

If a strain of bacteria is transplanted to a medium differing from the one in which it has been grown, a greater degree of adjustment occurs. For instance, coliform bacilli ferment both glucose and lactose, but if cultured continuously on glucose and then transferred to lactose, the growth rate, measured as the mean generation time, is reduced. After several transfers in lactose the rate increases, and the strain grows as rapidly in lactose as it formerly did in glucose. If the training to lactose is only partial, reversion occurs in nonlactose-containing mediums, but if the strain has been carried through many transplants in lactose, the ability to use the sugar with a maximum efficiency persists. Since the bacterium ferments both sugars with facility the adaptation is not overt, but it is, nevertheless, a typical adaptation, and the result is clearly a consequence of alteration in the balance of enzymes already existing in the cell. The adaptive process is much more striking, and less obviously a result of change in enzyme balance, when there is a great differential between the parent and adapted strains, as between no detectable acidity and an active fermentation, whether it occurs slowly on successive transfer or very rapidly as in the formation of an adaptive enzyme.

The change in enzyme balance may be quantitative in nature. The enzyme in question may be present only as a precursor, the formation of enzyme occurring in the presence of substrate through mass action as suggested for adaptive enzyme formation. Or the enzyme may be initially present in only very small amount, but it has a high turnover rate. Or, finally, catalysis of the new reaction may be a function of an existing enzyme but at a relatively low reaction velocity, and adaptation a matter of expansion of that enzyme. The last opens the question of qualitative modification of the enzyme, in that distortion of its specificity by a slightly different substrate requires greater activation energy and hence a lowered reaction rate, but if the substrate is present during formation of the enzyme, and the distortion not too great, a modified pattern might eventually result.

Returning to the example of the development of drug resistance by microorganisms it is as satisfactorily accounted for on the basis of an adjustment of bacterial enzyme systems as on that of a stepwise selection of resistant mutants, and here the nature of the resistance becomes significant. As indicated earlier, drug resistance may result from the production of an inhibitor such as p-aminobenzoic acid in excess amounts, a qualitative modification such that the drug displaces the antagonist as an essential metabolite, or by diversion to an alternate metabolic pathway. The first of these is clearly an expansion of an existing catalytic system. Regarding the second, suppose it be assumed that the drug displaces the prosthetic group of an enzyme, viz., pantolyltaurine, to give a modified enzyme and the formation of different metabolic products. The adaptation can thus consist of an altered enzyme balance to allow the metabolism of the new intermediates, and, when this is established, the original essential metabolite competitively inhibits the new system, and the drug has become an essential metabolite. The development of an alternate metabolic pathway is likewise an expansion of an existing enzyme or system of enzymes which ordinarily contributes in but small amount to the maintenance of the concentration of a given intermediate, perhaps because of lower reaction velocity. When the function of the usual system is inhibited by the drug, the alternate is expanded, perhaps only quantitatively, to neutralize the effects of the lower reaction velocity. The whole is analogous to an industrial system in which a raw material for a given process is shut off, an alternative, more expensive, process is developed, and the economies effected in large-scale operation make it as efficient as the original process.

There is considerable evidence in support of the view that the development of drug resistance is intimately related to the presence of the antibacterial substance which functions to distort metabolic processes^{92, 94} as well as to serve as a selective element in the environment. For example, resistance may develop in the presence of concentrations of the drug too small to inhibit growth, or the rate of development of resistance may depend upon the way in which the microorganism is exposed to it or be too rapid to be accounted for by selection of spontaneous mutants. Such evidence, too detailed for presentation here, is not only consistent with a relatively rapid adjustment of the bacterial enzyme systems to the new circumstances, but in some cases

hardly admits a different interpretation.^{28, 114} Similarly, the acquisition of drug resistance by viruses, as in the case of picornaviruses and guanidine described above, is difficult to account for on other than an induction basis against the background of the nature of viral replication.⁵⁴

Induction versus mutation. There is probably little or no justification for regarding specifically induced microbial variation as completely apart from, and competitive with, variation arising as a consequence of alteration in the genetic apparatus of the microorganism. Conversely, it is untenable to account for all microbial variations other than adaptive enzyme formation on a basis of spontaneous genic mutation and selection. Rather, it is self-evident that the observed variant is the phenotypic, and therefore environmentally dependent, expression of the microbial genotype.

In relatively few instances, notably that of the β -galactosidase-permease system of mutabile strains of coliform bacilli described earlier, can these be sharply distinguished. In the great majority of microbial variations the distinction is not clear, leading to uncertainty as to the nature, or more precisely the relative importance, of the mechanisms operative in the production of the observed variant. The induced variations combine with genic mutation to increase

the adaptability of the microorganism substantially beyond the limited range imposed by a one-to-one or similar relation between genic unit or subunit subject to spontaneous mutation and the production of, say, a given enzyme. By their occurrence, the induced variations make unnecessary the postulation of improbably complex polygenic controls.

The lack of clarity of distinction goes beyond that of interpretation of a given variation for, on the one hand, genic modification can be induced in the transduction reactions, and, on the other, metabolic sequences may behave in a unitary way suggestive of a genic nature. In connection with this last, Pollock80 has suggested that chain reactions controlled by adaptive enzymes may be cyclic in that the end product and the starting material are identical. Such self-reproducing biochemical cycles, or "genocycles," may well have some degree of autonomy at a subcellular level, with single unit cycles showing the characteristics of genes, and multiple unit cycles that of linked genic characters. Further, such a view includes the possibility of variation arising from intracellular competition between cycles through diversion of a component of one cycle to another or even the initiation of new cycles by rearrangement to give the appearance of genic mutation.

Microbial Phylogeny

Among the microorganisms, as among other living things, phylogenetic or evolutionary relationships are inferred from existing, *i.e.*, persisting, forms. At one time it was thought that bacteria were the first forms of life because only the autotrophic bacteria could live in an inorganic environment. It is hardly likely that a complete, highly organized cell, containing complex catalytic systems and having the broadest synthetic powers, could have appeared *de novo* in an inorganic world.

There is now excellent reason to believe that a chemical evolution, resulting in the appearance of organic matter, antedated the origin of life.^{12, 34, 76, 104} It was suggested by Haldane in 1926 that amino acids and heterocyclic compounds found in proto-

plasm could have been formed from single carbon compounds such as formaldehyde under the influence of solar ultraviolet light. The indicated experiments have been carried out by many workers and have shown that such organic compounds may be formed in this way. Some years later Oparin suggested that carbon was initially in the form of metallic carbide which, on contact with water, formed acetylene, and polymerization of the acetylene, catalyzed by mineral substances, could have resulted in the formation of long-chain carbon compounds which may, in turn, serve as precursors of the kinds of molecules found in living protoplasm. A third possibility is that of the formation of organic molecules from inorganic substrates under the influence

of high energy radiation, and this has also been substantiated by experiment. Finally, electrical discharges in the presence of substances such as methane, hydrogen, ammonia, and water, i.e., under reducing conditions, result in the appearance of a variety of compounds, particularly of the amino acid type; these reactions too are demonstrable in the laboratory. The appearance of such substances through any or all of these means is necessarily a random process, but as soon as a substance occurs that has catalytic activity, an element of selectivity appears, and a plausible case may be made for, for example, the chemical evolution of the iron porphyrin compounds. 16, 73

Presumably toward the end of this period of chemical evolution, self-reproducing substances appeared to constitute the beginning of the phenomena uniquely associated with life. It has been suggested by Haekel, Oparin, and more recently Salser,88 that the occurrence of autonomous biochemical cycles, perhaps analogous to the phenomenon of enzyme adaptation, preceded the development of the coding mechanism of replicating nucleic acids. It has been objected that a nucleic acid system is, in fact, not only a necessary, but a much more probable primitive system,74 especially in view of recent information on the formation of nucleotide chains in the absence of the living cell. However this may be, it is likely that the bacteria and blue-green algae were among the first forms of life organized on a cellular level. Of existing physiological types of bucteria, the facultative anaerobic free-living heterotroph would appear to most nearly resemble an ancestral form from which four main lines of divergence to more highly specialized forms may be distinguished.

A relative scarcity of metabolizable organic substrate would favor the evolution of the autotrophs, and these may have differentiated quite early, first as the facultative autotrophs and then the obligate autotrophs. The facultative autotrophs are characterized by a relative flexibility in their requirements for oxidizable substrate and retain to some degree the ability to respire anaerobically. The sulfur bacteria, oxidizing compounds of sulfur ranging from hydrogen sulfide to polythionates and able to live anaerobically

in the presence of nitrate, are representative of the group. Such bacteria may be regarded as transitional forms in the phylogenetic sense, giving rise on the one hand to the pigmented photosynthetic sulfur bacteria, and on the other to the obligate aerobic obligate autotrophic bacteria such as the nitrifying bacteria restricted to the oxidation of ammonia to nitrite or nitrite to nitrate.

Two lines of divergence occur with respect to the utilization of gaseous oxygen. One of them is that of the obligate anaerobes. highly specialized free-living heterotrophs closely adapted to living in the absence of oxygen, which lack a portion of the respiratory catalytic system, and characterized by unusual energy-yielding reactions such as the oxidation-reduction of paired amino acids and the metabolism of lactic acid to compounds such as butyl alcohol and acetone. The other line is that of the obligate aerobes which grow poorly or not at all in the absence of molecular oxygen, since they lack the ability to reduce many substances other than oxygen in the oxidation-reduction reactions of respiration.

The parasitic and pathogenic microorganisms may have been derived from these three groups of free-living heterotrophic bacteria. It is probable that toxigenicity in the broad sense is a prerequisite to the evolutionary development of the parasite in that it represents the entering wedge to invasion through deleterious effects on the integrity of the host tissues. The formation of potent toxins by certain of the free-living heterotrophs such as the botulinus bacilli, and the limited invasive powers of highly toxigenic bacteria such as the tetanus bacillus. suggest that they are forms which, though toxigenic, are not yet, or are only poorly, adapted parasites in that they are unable to invade the host tissues and set up a sufficiently mild infection so that they may persist in the host.

There appears to be little question that the host-parasite relation has markedly affected the microorganisms that became parasitic and contributed significantly to their phylogenetic differentiation. ¹⁰¹ For the most part, such effects are inferred from existing kinds of microorganisms and the diseases they produce, but in the case of syphilis the relative mildness of the modern disease as contrasted with its severity in

the early sixteenth century has been taken by some to be indicative of a reduced virulence of the spirochete for man.

If it be assumed that an important factor in the survival of the parasite is the extent to which it achieves a relatively innocuous but persisting infection in the host, from the microbial point of view the microorganism that produces a highly fatal disease may be said to have failed. It must be noted in this connection, however, that the causative agents of highly fatal diseases of man are not necessarily evolutionary failures; the high fatality of certain rickettsial diseases such as spotted fever, for example, is a consequence of adaptation to a host other than man, in this case the arthropod which is the vector from the anthropocentric point of view. To the microorganism the arthropod is the host, and human infection is an inconsequential episode in its life history.

The adaptation of the microorganism to the tissues of the host may be accompanied by an increasing dependence upon the host. Varying degrees of dependence are clearly evident as the generally fastidious nutritive requirements of the parasitic microorganisms and by host, and even tissue, specificities. In the first instance most pathogenic bacteria that occur within the host tissue proper require a relatively rich culture medium for growth and are often significantly altered by cultivation in vitro, as shown by loss of ability to invade the host. less exacting nutritive requirements, etc., as described earlier. Still others, such as the syphilis spirochete and the leprosy bacillus, have not been cultivated in vitro, and the rickettsiae and viruses are distinguished by their requirement for a living cell substrate for growth and a lack of other than token independent metabolic activity at the most. While the viruses are thought by some to be extremely primitive microorganisms, it is generally believed that they are degenerated forms occurring in continuous series from the rickettsiae, which serve as a connecting link to the bacteria, through the agents of the psittacosis-lymphogranuloma group, to the larger viruses of the pox group, and culminating in the viruses consisting only of nucleoprotein.4

In the second instance, a microorganism may be well adapted to some kinds of tissue but not to others. For example, the meningococcus occurs widely in the upper respiratory tract of man and is restricted to the human host, but when it invades the central nervous system, it produces a highly fatal disease. Similarly, infection with poliomyelitis virus is very common and confined to man, but only in the rare individual does it damage the host to give symptoms of the disease. The green streptococci are universally present in the mouth and upper respiratory tract of man, and their presence is regarded as "normal," but when they infect the valves of the heart, the subacute bacterial endocarditis produced is a highly fatal, though rare, disease. In effect, infection may be widespread but not overtly apparent, with actual disease occurring only rarely. Such a microorganism is a welladapted parasite and is subjected only rarely to the unfortunate happenstance of causing fatal disease.19

The extent to which the microbial variations are significant factors in a general pattern of evolutionary differentiation and specialization of microorganisms is a matter of speculation, but no more so than the role of classic genetics in the evolution of higher forms. At the same time, such variations are an important factor in the finer distinctions between microorganisms. Microbial phylogeny may be inferred with respect to broad tendencies and groups of microorganisms but apparently does not penetrate to the levels ascribed to microbial genus and species. For instance, when the antigenic structure of bacilli of the Salmonella group began to be precisely defined, a number of "trees" purporting to show a phylogenetic relationship among the antigenically different types were constructed. This is no longer of any interest because it has become clear that such types may be shown to occur by transduction and recombination, and it is highly probable that antigenic types are produced in nature in this way, particularly by phage-mediated transduction. From the phylogenetic point of view, the fertility groups, rather than subdivisions of them, are the important element, but as yet these are poorly understood.

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THE TAXONOMY OF **MICROORGANISMS**

Of the microorganisms, the fungi are clearly plants and the animal parasites are unicellular and multicellular animals, and both groups are formally classified on the conventional morphological basis as described elsewhere (Chaps. Thirty-two and Thirty-four). The bacteria and viruses occupy an intermediate position between the plant and animal kingdoms. They are linked to the fungi through the actinomyces and plant-like bacteria, especially the mycobacteria (tubercle and related bacilli) and the corvnebacteria (diphtheria and diphtheroid bacilli). Their relation to the animal kingdom through the protozoa is less clear. though the spirochetes are considered by many to be more animal-like than other bacteria. The rickettsiae and viruses are perhaps best regarded as offshoots of the bacteria and related to the plants and animals proper only through the bacteria.

Since the bacteria have properties of both plants and animals, at one time the question of whether they should be allocated to one kingdom or the other was an active one. An alternative proposal that gained some support, but not acceptance, was the creation of a new third kingdom, the Protista, for these forms. In time it became apparent that this question is irrelevant in the sense that an unequivocal answer to it serves no useful purpose, and the whole matter has been largely dropped from active consideration. Relatively early, however, the opinion prevailed that, in spite of the occurrence of both plant-like and animal-like properties among the bacteria, in total they resemble plants more than animals, and they were formally classified with the plants under Thallophyta as Schizomycetes, or fission fungi. The postulated relationship to other plant phyla is indicated in the accompanying outline. At the same time, the prominence of comparative physiology in the characterization and differentiation of the bacteria and the animal-like nature of their physiological processes leads many bacteriologists to think of them casually as animals and refer to them as "experimental animals." There is, of course, little or no basis for classification of the rickettsiae and viruses as plants, or animals either, for that matter.

Although the interrelationships of the organisms included under Schizomycetes present problems which are, in many essentials, new to the taxonomist, certain "natural" groups are apparent. Of these the most obvious is that based on morphology, with primary division into spherical, rod-

Phylum I. Thallophyta, plants without distinction of root, stem, and branch

Subphylum 1-the algae

Subphylum 2-the fungi-thallophytes lacking chlorophyll

Class I. Schizomycetes-the bacteria

Class II. Myxomycetes—the slime molds

Class III. Phycomycetes-the algae-like fungi

Class IV. Ascomycetes—the fungi which form ascospores Class V. Busidiomycetes—the fungi which form basidiospores

Phylum II. Bryophyta-the mosses

Phylum III. Pteridophyta-the ferns

Phylum IV. Spermatophyta-the seed-bearing plants

shaped, spiral, and filamentous forms, and subdivision on the basis of spore formation, presence and location of flagella, and staining reactions to the Gram and acid-fast stains. The earlier classifications, the better known of which are those of Migula and of Lehmann and Neumann, were made on this basis. These classifications exerted a strong influence on bacterial taxonomy and resulted in the naming of a great many species of bacteria, many of which persist.

Morphology is, however, not a sufficient basis for the separation of bacterial species, for many morphologically similar organisms may be quite different in other respects. Physiological differences, generally readily determinable in the laboratory, have been widely used. A number of workers are of the opinion that comparative physiology should constitute the primary basis of separation, rather than being subordinate to morphology.

Beyond such primary subdivisions, classification becomes increasingly difficult, and it is quite clear that this is so because not nearly enough is known of the phylogenetic relationships of the bacteria to one another for a detailed classification with definition of genera and species. This question has been discussed in some detail by van Niel,²³ who shows that as yet any classification can be little more than a key, and species are only "form" species, that is to say, no more than convenient handles.

The fundamental difficulty in preparing a classification rather than a differential key for the bacteria and viruses is a general uncertainty as to the significance or triviality of the differential characters used.6.10.19 Inevitably, a sound taxonomy will need to be based on microbial genetics and fertility systems, the latter in the broad sense to include the transduction reactions as well as sexual recombination. Still other approaches include the occurrence of key cellular constituents determinable by biochemical analysis; there is, for instance, some biochemical basis for Gram stain differentiation, evidence suggesting that content of amino acids and sugars, as determined by chromatography, and the purine and pyrimidine base ratios in microbial DNA show some correlation with genera and species, etc.

While the variability of microorganisms is significant to classification, it is not, as

might be supposed, a deterrent to differentiation and characterization by the usual physiological and immunological methods. Instability becomes evident when the environment is varied, but ordinarily microorganisms isolated in the same way retain their individuality for purposes of identification, or, if variation is common, as the O-D variation of influenza virus on primary isolation in the allantoic cavity of the embryonated hen's egg, it occurs with some regularity and is taken into consideration. Aberrant forms occur from time to time, of course, but in general an identification key is both practical and useful even though it may fail to represent genetic or other relationships.

Of the variety of formal classification systems prepared over the years, that of Bergey's Manual¹ has been the most detailed and has persisted, though it is not generally accepted. This, and other classifications have purported to be based upon phylogenetic relationships. It now seems to be generally, if tacitly, understood that microbial genera and species are no more than form genera and species. At the same time, there are practical problems of identification, as for diagnostic purposes, of some generally accepted common coinage of nomenclature. The former is recognized in the preparation of keys, such as that of Skerman²⁰ based on the Bergey classification, and those of Cowan^{4, 5} covering the pathogenic forms. The latter appears to be evolving in compromise form through the efforts of the International Association of Microbiological Societies.

Numerical taxonomy. 14, 21 With the general availability of computers, and the spreading conviction of the present futility of a phylogenetic classification of microorganisms, considerable interest has developed in numerical taxonomy. In general, unit characters are used and are not weighted, although complex characters broken down into units acquire an effective weighting. Such unit characters are necessarily derived from what are known as operational taxonomic units (OTU) which, in practice, are strains of microorganisms. Since no differentiation is made as to the relative "fundamental" importance of such unit characters, a classification derived from them is phenetic rather than phylogenetic. A relatively large number, not

Classification of Microorganisms According to Bergey (1957)

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ORDER	FAMILY	TRIBE	GENUS	
	Nitrobacteraceae		Nitrosocccus	The nitrifying bacteria
	Methanomonadaceae		Methanomonas	Methane bacteria The hydrogen bacteria
Pseudomonadales (suborder Pseudomonadineae)	Thiobacteriaceae		Thiospira	Sulfur vibrio Certain of the sulfur bacteria
	Pseudomonadaceae		Pseudomonas	Ps. aeruginosa et al. Acetic acid bacteria
Chlamydobacteriales	Spirillaceae		Vibrio Cellvibrio Spirillum	Cholera, paracholera, and noncholera vibrios Cellulose oxidizing vibrios <i>Sp. minus</i> (rat-bite fever) and saprophytic species Filamentous saprophytic bacteria, including sheathed iron bacteria
	Azotobacteraceae		Azotobacter	Nonsymbiotic nitrogen-fixing bacteria
			Rhizobium Agrobacterium Chromobacterium	Symbiotic nitrogen-fixing bacteria Plant pathogens and saprophytes Certain pigmented saprophytic bacteria
	Achromobacteraceae		Alcaligenes Achromobacter Flavobacterium	Alc. fecalis and related forms Nonpigmented soil and water bacteria Pigmented soil and water bacteria
		Escherichieae	Escherichia	Coliform bacteria Friedländer's bacillus
	Enterobacteriaceae	ErwinieaeSerratieae	Erwinia Serratia Proteus.	Plant pathogens Serratia marcescens (Chromobacterium prodigiosum) and related forms Pr. vulgaris and related forms
		Salmonelleae	SalmonellaShigella	Typhoid and paratyphoid bacilli Dysentery bacilli
Eubacteriales	Brucellaceae		Pasteurella	Plague and hemorrhagic septicemia bacilli Bacilli of undulant fever and contagious abortion Influenza bacillus, pertussis bacillus, chancroid bacilli Actinobacillosis bacillus, glanders bacillus
	Bacteroidaceae		Bacteroides	Nonsporulating obligate anaerobes

	Micrococcaceae	Staphylococcus Gaffkya	I he staphylococcc: M. tetragenus and related bacteria S. lutea and other cocci
	Neisseriaceae	NeisseriaVeillonella	Gonococcus, meningococcus, etc. Certain anaerobic cocci
	Streptococceae	Diplococcus	Pneumococcus and related forms The streptococci Saprophytic cocci
	Lactobacilleae	Lactobacillus	Lactic acid bacteria
	Propionibacteriaceae	Propionibacterium	Propionic acid bacteria
	Zorynebacteriaceae	Corynebacterium Listeria Erysipelothrix	Diphtheria and diphtheroid bacilli L. monocytogenes Bacterium of erysipeloid and swine erysipelas
	Bacillaceae	Bacillus	Aerobic spore-forming bacilli such as B. anthracis and B. subtilis Obligate anaerobic spore-forming bacilli, including bacilli of tetanus, gaseous gangrene, and botulism
	Mycobacteriaceae	Mycobacterium	Tubercle bacilli, leprosy bacilli, saprophytic forms
Actinomycetales	Actinomycetaceae	NocardiaActinomyces	Aerobic, sometimes acid-fast actinomycetes Anaerobic actinomyces of actinomycosis
	Streptomycetaceae	Streptomyces	Soil forms, often producing antibiotics
Spirochaetales	Spirochaetaceae	Spirochaeta	Free-living forms Saprophytic water forms Parasite of molluscs
	Treponemataceae	BorreliaTreponema	Relapsing fever spirochetes Spirochetes of syphilis and yaws Spirochetes of infectious jaundice and field fever
Mycoplasmatales	Mycoplasmataceae	Mycoplasma	Pleuropneumonia-like organisms (PPLO)
	Rickettsieae	Rickettsia	Typhus fever, spotted fever, etc. Q fever
Rickettsiales	Kickettsiaceae Ehrlichieae	Cowdria	Heartwater disease of cattle
	Bartonellaceae	Bartonella	Bartonellosis of man Bartonellosis of dogs, rodents, and cattle
Virales			Viruses infecting bacteria, plants and animals

less than 50, of unit characters must be used to give reasonable confidence limits to the results. The use of many units gives a cross-section of the group so analyzed, and a general purpose classification which is polythenic in character. When fewer units are used for the development of special purpose classifications, *e.g.*, diagnostic keys, the classification tends to be monothetic. Such special purpose classifications may be derived from a general purpose classification.¹²

Following tabulation of the data, i.e., n character units as distributed among t operational units (OTU), similarity indices (S) are calculated for each pair of OTU's. A commonly used measure of similarity is a coefficient of association, $S = n_s/(n_s + n_d)$ when n_s is the number of similarities, and n_d the number of differences. Tables of S are normally triangular. Cluster analysis is carried out by forming taxonomic groups of OTU's which show the greatest similarities. These clusters are called phenoms, and may be arranged in diagrammatic form based on their similarities to one another. Such analysis can lead to the definition of a "mean organism" or most typical strain and this can be considered to be the neotype, for example, a neotype of Pseudomonas.11

Other methods of analysis may be used. Principal component analysis avoids the preparation of cluster diagrams, and differs in that it gathers characters into clusters and weights them.⁸ The use of probabilistic similarity indices is a somewhat more lengthy procedure, but also avoids the clustering procedure, and is said to provide finer subdivisions.⁷

Nomenclature.^{3, 9} Neither the botanical nor zoological codes of nomenclature have been satisfactorily applicable to the bacteria and viruses, and an official International Code of Nomenclature of Bacteria and Viruses has been, and continues to be, developed by committees appointed by the several International Congresses of Microbiology.

In general a bacterium has a generic name, always written with a capital letter, which may or may not be descriptive (for example, Bacillus—a small rod; or Pasteurella—in honor of Pasteur) and a specific name which may be an adjective (albus—white) or a noun indicating possession (Clostridium welchii—Welch's clostridium) or a noun in apposition (Bacillus radicicola—the root-

dweller bacillus). The practice of indicating the author of the name, as in Bacillus subtilis Cohn, is not as common in bacteriology as in zoology or botany. The use of trinomial and quadrinomial names such as Granulobacillus saccharobutyricus mobilis nonliquefaciens is obviously highly undesirable. Trinomials are occasionally useful in the designation of varieties of subspecies. Common names are, of course, used; terms such as Friedländer's bacillus and the typhoid bacillus are frequently encountered.

Genera. Perhaps one of the greatest difficulties the bacterial taxonomist labors under is the paucity of genera. The question of what degree of difference shall be judged sufficient to establish new genera is one to which there is as yet no satisfactory answer. At the present time considerable confusion and lack of uniformity still exist. In a number of instances new generic names have obtained wide currency among bacteriologists in many parts of the world, partly, doubtless, because they are applied to fairly distinct groups of microorganisms and have genuine classificatory value. Such are Brucella for the bacilli of undulant fever of man and contagious abortion of cattle, Salmonella for the paratyphoid bacilli, and Pasteurella for the bacilli of hemorrhagic septicemia in domestic animals and the bacilli of plague and tularemia; the name Shigella for the dysentery bacilli has gained a considerable degree of recognition. Nevertheless, the status of genera of bacteria, and viruses especially, is questionable in that they do not have the status of botanical and zoological genera based on character or gene frequencies and capacity for hybridization. The cross-fertility shown by recombination between Escherichia and Shigella and Salmonella referred to earlier (Chap. Seven) is a clear indication of the uncertainties in definitions of bacterial genera.

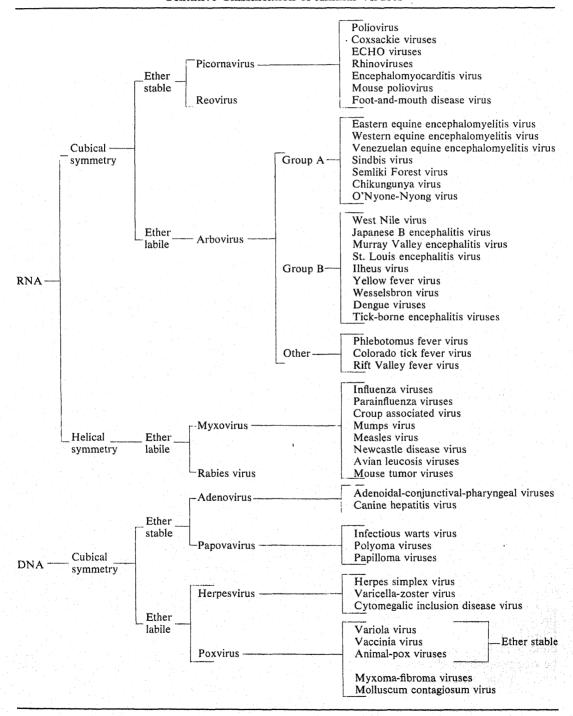
Species. 16 The differentiation of species is made for the most part on a physiological basis. A single character is hardly sufficient, particularly in view of the facility with which variation occurs. The use of a number of such characters is the rule, but difficulties are frequently encountered when intermediate forms occur. This is common among the enteric bacilli, possibly because these are better known than many groups of bacteria.

Types.² Immunological differentiation is generally regarded as not of species status though it may sometimes coincide with spe-

cies differentiation on a physiological basis. Conversely, minor cross-reactions between species are not indicative of species identity. There are some exceptions to the former, however, and many workers divide the genus Salmonella into species on the basis of antigenic structure. Immunological differences

are of very considerable value, particularly for epidemiological purposes, and are usually the basis of distinction of types within a species. Thus the streptococci are divided into groups, designated A, B, C, etc., on the basis of one kind of antigen, and into Arabic-numbered types within and across species by

Tentative Classification of Animal Viruses



another. The pneumococci are similarly divided into numbered types on the basis of capsular antigen. Clostridium botulinum is separated into types, A, B, C, etc., on the basis of the immunological specificity of the toxin, and these have no relation to the immunological character of the cell substance. There is, then, no general practice with respect to type differentiation. Physiological differences within species are usually not used to differentiate types, and the biochemical reaction is simply given as variable.

Classification of viruses. 13, 15, 24 While the classification of viruses presents problems to the taxonomist that are in many ways unique, the questions of characterization and nomenclature require answers. As in the case of the bacteria, a number of names have been evolved or suggested that have proved useful and generally acceptable. Terms such as poxvirus, poliovirus, and herpesvirus are simply a contraction. Others, such as adenovirus and rhinovirus were suggested as such. Still other names are descriptive terms for groups of viruses. The last includes reovirus as indicative of their respiratory-enteric origin, picornavirus meaning small (pico) RNA viruses, arbovirus (or arborvirus) to designate the arthropod-borne viruses, and papovaviruses for those of the papillomapolyoma group.

Dichotomous separations may be made on the basis of a number of characteristics. leading to definition of groups of viruses. A primary separation is effected on the basis of nucleic acid, i.e., into DNA and RNA viruses. Similarly, symmetry, helical or cubical, allows a second dichotomy. A further useful separation may be made with. respect to ether sensitivity, and may reflect a basic difference in lipid structure of viruses. The presence or absence of a limiting membrane, or envelope, makes possible still another dichotomy.17 More detailed characteristics, such as the diameter of the nucleocapsid of helical viruses and the number of the triangulation and number of capsomeres in the capsid of cubical viruses, have been suggested to allow further differentiation. 18

Separation into main groups, as shown in the accompanying diagram, seems to be both feasible and useful, and viruses whose nucleic acid type and symmetry have yet to be determined should fall into such a tentative schema. Whether or not elaboration to a detailed classification, necessarily requiring additional new and unfamiliar nomenclature, as has been proposed¹³ would be more confusing than revealing at the present time is not clear.

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Chapter Nine

THE PATHOGENIC MICROORGANISMS AND DISEASE

Infectious disease may be regarded as a by-product, and not an inevitable one, of the host-parasite relationship. While, as suggested earlier, the initiation of the career of a parasitic microorganism may be based on its ability to produce sufficient damage to the host to facilitate the development of foci of infection, pathogenicity is incidental to the parasite and has significance only as it relates to the establishment of an ecological niche and to the survival of the microorganisms.

The etiological relation of microorganisms to infectious disease was suspected by some not long after their discovery by Leeuwenhoek and, in a sense, found support in Jenner's vaccination studies and those of others, especially the classic work of Snow on the transmission of cholera, in which a con-

tagium vivum was implied. It was not until past the middle of the nineteenth century that, as described elsewhere (Chap. One), this belief began to crystallize into knowledge through parallel developments such as the transmission of anthrax by infected blood by Brauell in 1867 and the recognition of the resemblance of fermentation processes to sepsis to provide the basis of Lister's antiseptic surgery, culminating in Koch's demonstration of the microbial etiology of anthrax. Through exploitation of his pure culture techniques in application to a wide variety of diseases, the etiology of many was resolved to displace the Hippocratic doctrine of imbalance of the four bodily humors-blood, phlegm, yellow bile, and black bile-as the basis of all disease.

The Specific Microbial Etiology of Infectious Disease

It is one thing to suspect, or even have some evidence of, the ability of microorganisms to produce disease and quite another to develop unequivocal proof of a specific etiological relation between a given microorganism and a given disease.

Koch's postulates. Such proof was first offered by Koch in the form of a logical chain of experimental evidence, usually known as Koch's postulates, although there is no evidence that they were so formulated by Koch, and good reason to believe that they originated with Koch's professor, Cohn. However this may be, there are four of these; a fifth was suggested at one time but it is usually not applicable.

The first postulate. The first of these is the observed occurrence of the microorganism in association with the disease. This is relatively simple in some diseases in which the causative microorganism is found in pure culture in some kinds of specimens, viz., the typhoid bacillus in the blood or the plague bacillus in fluid aspirated from the infected lymph nodes in the bubonic form of the disease. In others, however, notably infections of the respiratory and intestinal tracts, the causative microorganism occurs admixed with many other, often similar, forms and needs to be sorted out.

The requirement for such preliminary observation is self-evident in that if an associ-

ation were not observed there would be no reason to suspect an etiological relation requiring further investigation. At the same time, it is necessary to emphasize the first postulate for a number of reasons. First, closely similar, even indistinguishable, clinical syndromes may have quite different etiology. For instance, it is not possible to distinguish among certain diarrheal diseases of varied etiology on clinical grounds alone, e.g., acute bacillary dysentery and cholera occurring, as often is the case, in debilitated persons; a variety of bacteria may be the cause of urinary tract disease; and the problem of the etiology of the so-called common cold is largely a consequence of a wide variety of etiological agents which give rise to substantially the same symptoms.

Second, the term "observation" must be taken broadly. In the case of pathogenic bacteria there may be no morphological distinction between the pathogen and related nonpathogens, e.g., bacteria such as the diphtheria and diphtheroid bacilli and the pathogenic enteric bacilli and nonpathogenic coliforms are morphologically indistinguishable. Further, when the infectious agent is too small to be resolved in the light microscope, as is the case with the viruses, it cannot be directly observed. Thus the first postulate melts into the second in that characterization and differentiation of the microorganism in isolated form are often, in fact usually, required to establish its presence in association with the disease.

Finally, the first postulate has been taken to imply not only presence of the microorganism in association with the disease, but also the corollary that it is absent in the absence of disease. Quite aside from the occurrence of similar microorganisms in the same specimen, only one of which may be related to the disease, the pathogenic microorganisms may be present in individuals who have no symptoms of the disease, viz., inapparent casual infections, carriers, and other forms of asymptomatic infection. It was the presence of diphtheria bacilli in normal as well as diseased individuals that led Klebs, who discovered this microorganism, to question its etiological relation to the disease.

So, in practice, it is necessary to qualify the dogmatic first postulate in a number of ways, viz., observation must be stretched to include, for example, the cytopathology of tissue culture inoculated with infectious material, an observed association in at least a majority of cases of the disease, and regard microorganism as suspect even though it may be found in some healthy persons. Such association is by no means proof of an etiological relation, and further evidence is required.

The second postulate. The second postulate is that the microorganism must be isolated and grown in pure culture. This is clearly essential to effect a separation of the causative agent from other microorganisms when they are present and, as indicated above, is often an integral part of the first postulate for purposes of characterization of the agent. It is usually possible to isolate and grow in pure culture pathogenic bacteria, the practical difficulty being the requirement for an appropriate culture medium, but occasionally this cannot be done. The leprosy bacillus, regularly found in enormous numbers in material from lesions, is morphologically distinctive both in its acid-fast staining character and the arrangement of the individual cells but has not, despite perennial reports to the contrary, been cultivated in the laboratory, either in lifeless mediums or in tissue culture, nor has direct inoculation of leprous material been unequivocally successful. There is an immune response in the disease, manifested as a hypersensitivity to soluble material (lepromin) extractable from leprous nodules. This last is feeble supportive evidence because the hypersensitivity crosses with that to tuberculin, but the leprosy bacillus is provisionally accepted as the etiological agent of the disease, largely because there appears to be no alternative agent.

The spirochete of syphilis also has not been cultivated in compliance with the second postulate, but its etiological relation to the disease is more firmly established because supportive evidence is relatively strong. This includes a specific immune response to the spirochetal substance appearing during the course of the disease and the reproduction of the disease by direct inoculation of infectious material containing, so far as is known, only the spirochete. In some instances, then, the requirement of the second postulate may be circumvented with a greater or lesser degree of probability of the presumed etiological relation.

In the case of the rickettsiae and viruses,

isolation is necessarily growth in tissue culture or the embryonated hen's egg. It might be supposed that the purity of such cultures is questionable but, while this may be true to some extent, the occurrence of viruses in mixed culture in tissue culture is usually evident. Growth of these agents is directly demonstrable in the case of the rickettsiae. which may be seen in the light microscope. and indicated in the case of the viruses by appropriate, often characteristic, pathology of the tissue substrate, or some unique feature such as the development of hemagglutinin in the allantoic fluid of the embryonated hen's egg infected with viruses of the influenza group. In some instances, however, there may be no overt evidences of growth, and the agent may be carried by so-called "blind passage" in a series of tissue or egg cultures. After several such transplants cytopathic effects may become apparent, and in any case the material is tested for infectivity when the dilution is sufficient to rule out persisting infectivity. The somewhat devious nature of such procedures does not invalidate them.

The third postulate. The third postulate requires that the disease be reproduced in a susceptible animal by inoculation with the microorganism in pure culture. This is often difficult to accomplish for two reasons. First the disease must be reproduced in some recognizable form, i.e., will need to bear sufficient relation to the naturally occurring disease. Second, a susceptible animal may be difficult to find. These are not matters of great importance in the study of diseases of, for example, domestic animals, for the natural host may be the experimental host also. In the case of infectious diseases of man, however, some experimental animal must be found. The etiological agents of human disease are often closely adapted parasites, so much so that they will either not infect, or will not produce disease in an experimental animal that is recognizable as related to the naturally occurring human disease.

While in a general way certain of the monkeys and higher apes, such as chimpanzees, are more susceptible to infection with human pathogens than are, for example, rodents, susceptibility does not necessarily parallel phylogenetic relationships. The search for a suitable experimental animal becomes in large part a matter of trial and error and educated guess. In the 1930's, for instance, the only reasonably available experimental animal in which poliomyelitis could be reproduced was the rhesus monkey. One eminent investigator systematically inoculated all kinds of experimental animals that came to hand, and finally found that one strain of this virus could infect the cotton rat. Similarly, influenza could not be reproduced in experimental animals for many years until the similarity of the lesions of the lung produced in ferrets by distemper virus to those of human influenza was noted, and it was found that human influenza could be reproduced in the ferret by intranasal inoculation.

Other diseases of man, such as gonorrhea and typhoid fever, have not been reproduced in ordinary experimental animals, but some, such as tuberculosis and diphtheria in the guinea pig, are reproduced with facility. The mouse is exquisitely susceptible to infection with the pneumococcus on parenteral inoculation, and man appears to lie midway between the mouse and the more resistant dog with respect to susceptibility to infection with this bacterium. While there are many others, these examples suffice to illustrate the somewhat erratic occurrence of susceptibility to infection with the pathogenic microorganisms.

The importance of reproducing infectious disease in an experimental animal cannot be overestimated. Unless the disease can be studied under the controlled conditions of the laboratory it must remain inadequately understood. It is equally obvious that unless this third postulate is fulfilled it is not possible to fully establish the etiological relation of a given microorganism to a given disease.

The fourth postulate. According to the fourth postulate, the microorganism must be found in the infection produced in the susceptible animal, and shown to be the same as that inoculated. If the first three postulates are met, the fourth ordinarily follows without difficulty. It is nonetheless necessary to show that the animal was in fact infected with the microorganism and that the disease or death so produced is specific in that it is a consequence of that infection rather than some intercurrent infection of the experimental animal. Rarely there is some difficulty in this connection. Certain of the tumors of virus etiology, for example, are transmissible by cell-free filtrates, but after several passages by tissue implantation it may not be possible to find the virus. The reasons for this are relatively complex and, in part at least, are technical in nature.

The "fifth postulate." Following the discovery of the bacterial exotoxins, it was suggested that a fifth postulate, namely that the disease should be reproduced in susceptible animals by inoculation of the cell-free products of microbial growth, might be added. This is applicable in only a minority of cases and, while it lends support to the identification of the specific etiology of a disease, is not essential to establishing that etiology beyond reasonable doubt.

Supporting evidence. Less direct but supporting evidence for the etiology of a given disease assumes significance when the evidence provided by the four postulates is equivocal. This has been implied above in relation particularly to inability to reproduce human disease in characteristic form in experimental animals. The most important of such supportive evidence is immunological in nature. Taking the example of typhoid fever, and neglecting the accidental human infections with pure cultures of the typhoid bacillus which satisfy the requirements of the third postulate, immunological evidence strongly supports the etiological relation of the microorganism to the disease; the disease may be prevented by immunization with vaccines prepared from cultures of the microorganism, and the naturally infected individual who recovers from the disease develops an immunity directed specifically toward the microorganism which is demonstrable in a number of ways. Or the indirect supporting evidence may be epidemiological in nature, serving to pinpoint a particular microorganism with respect to a given disease even though the disease cannot be reproduced experimentally.

Multiple etiology. In naturally occurring disease more than one kind of microorganism may be concerned, and three kinds of such diseases may be distinguished. Under some conditions secondary infection is not uncommon, the primary invader serving to reduce the resistance of the host so that a second kind of microorganism, of limited pathogenic powers and often a part of the normal microbial flora of the host, may produce disease. Staphylococcal pneumonia, for instance, is usually not a primary infection but is found to occur most often as a secondary infection, commonly as a sequel

to influenza. Similarly, Proteus is of relatively limited pathogenic capabilities, but is potentiated by staphylococci, apparently a substance formed by staphylococci, so that it becomes a virulent, lethal agent.² Other kinds of diseases are characteristically mixed infections in that more than one kind of microorganism is concerned from the outset; thus, perhaps half a dozen species of bacteria are the causative agents of gaseous gangrene, occurring in various combinations but rarely alone. In both secondary and mixed infections the causative microorganisms are independent and individually capable of producing a disease condition.

The third kind of infection is that in which the etiological agent is not single but multiple, and the disease is a consequence of a synergistic relation between the microorganisms such that the combination has pathogenic capabilities that its components do not. Such multiple etiology becomes complex and is possibly more common than now appears. Two examples will suffice to illustrate it.

One is the now classic example of dual etiology of disease, that of swine influenza described by Shope. Here the two microorganisms are a porcine variety of the influenza bacillus, Hemophilus suis (H. influenzae var. suis), and a variety of the influenza virus. The disease swine influenza is not caused by either the virus alone or the bacterium alone. but only by a combination of the two. The other example is that of diphtheria of man. For 70-odd years there was no reason to believe that the microbial etiology of this disease was other than clearly and fully established. Not only was the diphtheria bacillus isolated and characterized in 1883, but the following year the powerful exotoxin of the microorganism, capable of producing the disease symptoms and pathology, was described. Subsequently antitoxin therapy and highly effective immunization with toxoid were developed. About 1950, however, it was found that only lysogenic diphtheria bacilli are toxigenic, and therefore pathogenic (Chap. Seven), and it became clear that the etiological agent of diphtheria is, in fact, not a single microorganism but a combination of the bacillus and the appropriate bacterial virus.

It is not yet clear how common the phenomenon of true multiple etiology may be. There are a number of indications that the etiology of many of the infectious diseases

may not be as firmly established as has been thought. For example, there seems to be little doubt of the pathogenicity of the hemolytic streptococci, and yet inoculation of human volunteers with freshly isolated cultures has often given negative results. Similarly, meningococcus meningitis was an important problem in military personnel during World War I but failed to materialize

to a significant degree during World War II in spite of high rates of carriage of the microorganisms. The occurrence of infectious disease is a highly complex phenomenon, involving more than the nature and properties of the causative microorganism, but it is possible that observed discrepancies may be due in part to an oversimplification of its microbial etiology.¹⁰⁹

Microbial Virulence 117, 121

The term "pathogenicity" has been used above in the generally accepted sense of ability to produce disease. It varies widely, not only from one kind of microorganism to another, but also between strains of a single kind of microorganism. A number of other terms denoting disease-producing ability are used, the most common of which is virulence. Here the terms "pathogenicity" and "virulence" are used in the sense suggested by Miles,78 viz., pathogenicity is taken to mean potential ability to produce disease and is applied to groups or species of microorganisms, while virulence is used in the sense of degree of pathogenicity within the group or species. In this way, it is possible to speak of avirulent, virulent, highly virulent, etc., strains within groups or species of microorganisms that are said to be pathogenic.113

It is self-evident that ability to produce disease on the part of a microorganism is expressed only when the disease is produced. Conversely, the resistance of the host (considered in the following section) is determinable only as a reaction to appropriate exposure to the microorganisms. While, then, the two are fundamentally inseparable, for practical purposes they may be measured by holding one relatively constant and varying the other.

Measurement of virulence. The measurement of virulence, then, consists of testing for ability to infect, with observable consequences, "normal" animals such as a standard strain of mice. The consequence observed is ordinarily an all-or-none phenomenon such as death. On challenge inoculation of experimental animals with graded doses of the microorganism, or substances such as bacterial toxins, it is apparent that virulence (or toxicity, etc.) is inversely re-

lated to the size of the effective dose, *i.e.*, the smaller the dose required to kill, for example, the greater the virulence. This is the basis of measurement of virulence (or toxicity) by determination of the minimum lethal dose, the smallest dose required to kill a standard experimental animal.

If each dose is given to groups of experimental animals, ranging in size from a minimum of four to a practical maximum of 10 to 20, rather than individual animals only, two additional points are apparent. First, the proportion of animals reacting in each group varies from none or practically none in the group of animals receiving the smallest dose, to all or practically all of the animals in the group receiving the largest dose. The notation is usually fractional, the numerator the number of animals reacting and the denominator the number of animals inoculated with that dose, and recorded as 0/6, 1/6, 3/6, and 6/6, for example, for the several amounts of challenge inoculum.

Second, when the groups of animals are sufficiently large and the doses graded appropriately, it is apparent also that the proportion of animals affected per unit increment of dose rises slowly at first, then rapidly, and finally slowly again as the proportion becomes large. If the cumulative deaths or other effects are plotted against the logarithms of the doses, the points tend to fall on an S-shaped curve which represents the integral of the frequency distribution of natural resistance in the experimental animals used. When the cumulative effect is plotted on probability paper, on the scale representing the integral of the normal frequency distribution, against the logarithm of the dose, the points tend to fall on a straight line which may be fitted by inspection or by least squares.

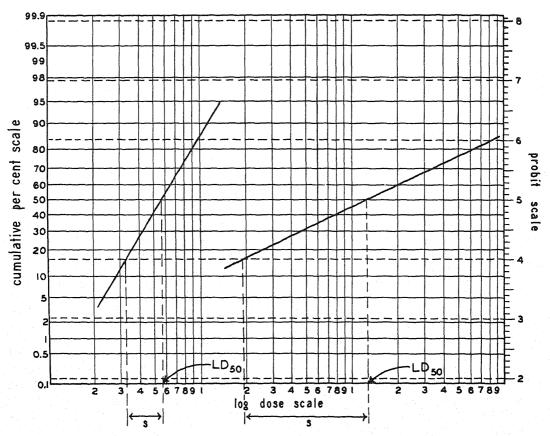


Figure 56. The graphical determination of the interpolated 50 per cent dose (ED_{50}, LD_{50}) and its standard error, s, on probability log paper. The vertical scale is the integral of the normal frequency distribution as per cent cumulative effect, and the horizontal scale is dose. The probit scale is on the right. Steep and less steep dose-response curves are shown.

The ED_{50} . The point on the dose scale at which this line intersects the 50 per cent point in the cumulative effect gives the interpolated dose which would produce the effect in half the animals inoculated with it. This is the 50 per cent effective or ED₅₀ dose, or LD₅₀ dose when death is the response measured. This value is, of course, subject to sampling error, and its precision is indicated by its standard error which is determined graphically also as indicated in the accompanying figure and is written as the $ED_{50} \pm$ the standard error. Such data allow the calculation of the significance of differences between titrations, as, for example, in protection tests (see below).7,8

The standard error is the standard deviation or standard deviate of the normal frequency distribution and is the distance,

in this instance on the dose scale, from center of the distribution to the point of inflection on either side and may, therefore, be either positive or negative to the central point. Since half the deviates are negative, the use of negative numbers may be avoided by adding the number 5; i.e., at the central point the value is 5, at -1 it is 4, at +1 it is 6, and so on. This altered deviate is known as a probit. When the probit values for the cumulative percentage mortalities, or other effects, are plotted against dose on a logarithmic scale the relationship is linear because the probit scale is another way of representing the integral of the normal frequency distribution. The ED₅₀ is the point on the dose scale at which the line fitted by inspection, or more precisely by the least squares method if appropriate, intersects

5 on the probit scale. The probit scale is superimposed on the probability scale in the accompanying figure.

The ED₅₀, or LD₅₀, measure of virulence is subject to qualification in that virulence is also indicated by the slope of the dose response curve. Slopes may vary widely from one pathogenic microorganism to another. When a highly virulent strain of pneumococcus is titrated in the mouse, for example, the dose response curve is steep and usually does not extend beyond a range of two logs on the dose scale. By contrast, when the virulence of Salmonella is titrated in the same animal, the dose response curve is much less steep, extending over as much as six logs on the dose scale. The significance of slope is not clear, but it is obvious that, to continue with the same example, the virulence of pneumococcus and that of Salmonella are not completely comparable as their respective LD₅₀ values alone.

The dose-response curve, or the increasing proportion of deaths as the dose is increased, may be considered to be due to the independent activity of the self-reproducing particles, i.e., to the increasing probability that a single effective microorganism will multiply to a point at which death occurs. Quantitative studies substantiate this in highly susceptible animals, i.e., when the LD₅₀ is very small, but in partially resistant animals for which the LD₅₀ is high, the total number of bacteria at death appears to be a consequence of growth of all the organisms inoculated rather than that of some effective portion.74, 75, 79 The latter is consistent also with the generally observed inverse relation between size of inoculum and length of the incubation period.

Mucin. Many of the microorganisms pathogenic for man are so closely adapted to the human host that they are relatively avirulent for the usual experimental animals. It was first observed by Nungester that the virulence of many such microorganisms, e.g., meningococcus, typhoid bacillus, dysentery bacilli, the cholera vibrio, can be markedly enhanced by inoculation of the bacteria suspended in gastric mucin. The role of mucin appears to be that of protecting the microorganisms against phagocytosis in the usual laboratory titration, but respiratory and alimentary mucus may function to protect the mucosae against infection in naturally occurring infections. Fractionation of this activity of mucins has been of some interest and a series of fractions have been obtained.35 These include a heparin fraction which is also anticomplementary and associated with polysaccharide A, a viscosity fraction which is also a polysaccharide (polysaccharide C) fraction which acts synergistically with the heparin fraction, possibly affecting capillary permeability, and a particulate residue fraction which also acts synergistically with the viscosity fraction, but independently of the heparin fraction. Mucopolysaccharides of other origin, especially bacterial levans, 22, 107 also show some enhancement of virulence, presumably by lodgment in the interstices of capillary endothelium, thus modifying permeability and the inflammatory response.

The enhancement of bacterial virulence by mucin and similar substances, while of very considerable importance in making possible experimental infections otherwise obtainable only with great difficulty, derives its greatest significance from its implications concerning relevant facets of natural resistance.

Infections induced in this way are sufficiently artificial that, when they are used to assay the immune response by either the active or passive method, they give only results open to serious criticism. Obviously the protection demonstrable with such infections by antibody, chemotherapeutic drugs, etc., is a protection of the animal against the effects of mucin as well as against the microorganism.

Protection tests. The virulence titration is often used to assay the efficacy of some treatment such as active or passive immunization, or administration of drugs, by comparing directly or indirectly, the LD₅₀ of a given microorganism for normal animals and that for treated animals. From the analytical point of view, i.e., the design of experiments, more reliable results may be obtained by using a standard challenge inoculum and varying the treatment. In measuring the protective capacity of immune serum by passive immunization of the animals to be inoculated, for example, it is preferable to administer varying amounts of antiserum to groups of animals, followed by challenge with some constant number of microorganisms. Similarly, in the active immunization test for the immunogenic potency of vaccines, groups of animals are given graded

doses of vaccine followed by a constant challenge dose of bacteria after sufficient time to allow the immunity to develop. This preferred procedure is not always possible when the virulence of the challenge microorganism varies widely from one time to another. In such cases the protective material is given in constant dosage, and the LD_{50} dose is titrated in both treated and control animals, and protection measured by the ratio of the LD_{50} in treated animals to that in control animals.

The neutralization test, or serum neutralization test, is a variation on the protection test which is applied to the titration of antitoxin and antibody to viruses. The toxin or infective virus preparation, previously titrated so that the dose is known, is mixed with varied amounts of antiserum, incubated for a time, and the mixture inoculated into susceptible animals. Survival of the animals is indicative of the presence of neutralizing antibody in the serum.

MICROBIAL TOXINS

The derangement of the normal physiological processes that is disease is, in the case of the infectious diseases, a consequence of the toxicity of proliferating microorganisms in the tissues. Toxic substances affecting tissues, cells, and possibly enzyme systems are formed by the microorganisms incidental to their metabolic activities which diffuse more or less freely from the microbial cells, and the cell substance of a wide variety of microorganisms is toxic also. In addition, toxic effects may be produced indirectly, as by the activation of tissue enzymes by bacterial kinases, or the formation of inflammatory and similar toxic substances by the affected tissues, and by the development of hypersensitivity (Chap. Fourteen) to the microbial cell substance.

The toxicities of microbial origin may be separated into three groups, the potent exotoxins, which are the classic bacterial toxins; the endotoxins contained within the intact cell; and a heterogeneous group of toxic substances, often enzymatic in nature, which diffuse more or less freely from the intact cell. Such a separation is artificial, in that lines of demarcation between the groups are often indistinct, but is useful for purposes of discussion.

EXOTOXINS^{51, 69}

The bacterial exotoxins are the most potent poisons known, and it has been calculated that as little as 7 ounces of crystalline botulinum type A toxin would suffice to kill the entire human population of the world. These toxins have their counterparts in the less potent zootoxins such as the snake, spider, and scorpion venoms, and in the phytotoxins such as ricin and abrin. The formation of exotoxins by bacteria is relatively uncommon and is confined to the botulinus bacilli, the diphtheria bacillus, the tetanus bacillus, and one species, Shiga's bacillus, of the dysentery bacilli. The gaseous gangrene bacilli are sometimes regarded as producing exotoxin, but these activities are multiple in that the same strain of bacteria usually produces more than one toxicity, and these activities tend to fall into the group of enzymatically active toxicities.

Crystalline type A botulinum toxin is the most potent of the bacterial toxins; the LD_{50} for the mouse is only 4.5×10^{-9} mg. N. Type B botulinum toxin is somewhat less potent, 5 to 9 \times 10⁻⁹ mg. N/LD₅₀ for the mouse, and crystalline tetanus toxin 6.6 × 10⁻⁷ mg. N/LD₅₀. Highly purified but noncrystalline preparations of diphtheria toxin kill guinea pigs in amounts of $0.4 \mu g$, per kilogram of body weight. The Shiga neurotoxin is of the same order of toxicity in purified form, but relatively small amounts are formed so that crude culture filtrates are not as toxic as those of the botulinus, tetanus, and diphtheria bacilli. While these amounts of toxin are extremely small, the toxins are neurotoxins and are not to be considered as diluted in the entire animal; the lethal dose of botulinum type A toxin for the mouse, for example, is about 20 million molecules, corresponding to about eight molecules per nerve cell.

The potency of these toxins is, in general, paralleled by their efficiency as antigens. Antitoxic serums of high titer, 1 ml. of which will neutralize thousands of guinea pig MLD's may be obtained against diphtheria toxin. The neutralization of exotoxin by antitoxin proceeds according to the law of multiple proportions, i.e., if x units of antitoxin neutralize y units of toxin, nx units of antitoxin will neutralize ny units of toxin.

The exotoxins appear to be proteins; they are denatured by heat, may be salted out of

EXOTOXINS 267

solution etc. The high-molecular-weight botulinum toxins (ca. 1× 10⁶) appear to be polymers and may be broken down into active fragments. Such active fragments of type A and type E toxins have molecular weights of 12,000 to 16,000,^{36, 37} and those of type B toxin a molecular weight of 9000 to 10,000.³⁸ Tetanus and diphtheria toxins have also been prepared in crystalline form, but have not been broken down to smaller active fragments. Diphtheria toxin appears to be a complex, at least antigenically, for multiple antigens are demonstrable by gel diffusion.

With the exception of botulinum toxin, the exotoxins are destroyed by proteolytic enzymes; botulinum toxin is, therefore, the only toxin which is effective when given by mouth.⁶⁰ Dispersion of the polymer by gastrointestinal proteases to more readily absorbable, smaller active fragments may well play a highly significant part in contributing to toxicity by the oral route. There is no chemical evidence which explains, even in part, the toxicity of these substances: these proteins do not seem to differ in any essential respect from bland proteins such as egg albumin. The amino acid composition of crystalline type A botulinum toxin, for example, is in no way unusual. It has been suspected that toxicity might be a property of a prosthetic group attached to the protein molecule, but this appears not to be true; the balance of evidence indicates that the toxicity is a property of the structure of the toxin molecule, possibly of the arrangement of the constituent amino acid molecules in the protein.

The pharmacological action of the soluble toxins resembles that of the vegetable alkaloids and is sometimes quite definite and characteristic for each toxin. Diphtheria toxin, for example, produces degeneration of the heart muscles, kidneys, and liver and the hemorrhagic reaction in the adrenals which is a highly characteristic postmortem finding in the guinea pig.

The nature of the toxicity which results in these changes is not clear, but in this connection the implied nature of the toxin is of considerable interest. Largely through the work of Pappenheimer, considerations of the iron and porphyrin metabolism of diphtheria bacilli in relation to toxin production (Chap. Thirty) strongly suggest that diphtheria toxin may be the protein moiety of

the cytochrome b of the diphtheria bacillus. This raises the interesting possibility that its toxicity may be related to the functioning of the respiratory enzymes of the host cells.

The activity of botulinum toxin has been studied in some detail. It has been found that acetylcholine produces contraction of botulinum-poisoned muscle but not of curare-poisoned muscle. This has been taken to indicate that acetylcholine is not produced in the end plates by the poisoned animal, and the action of the toxin is proximal to the point at which it is produced. Consistent with this, no acetylcholine is released during the neuromuscular paralysis produced by the toxin. The site of action of the toxin is on the nerve filaments, since acetylcholine is released following direct stimulation of the excised diaphragm of the guinea pig, but not following tetanization of the phrenic nerves. The neuromuscular paralysis occurring in the poisoned animal is a result of interference with conduction in the terminal twigs of motor nerves, near or at the points of final branching, but proximal to the site of acetylcholine release. The nature of the activity of the toxin that produces such interference with conduction is, however, completely unknown.

Tetanus toxin appears to act both centrally and peripherally. Its action on the central nervous system appears to be similar to that of strychnine, with suppression of synaptic inhibition. The peripheral effect seems to be two-fold and includes a spastic action at the neuromuscular junction of voluntary muscles (local tetanus) and a paralytic action similar to that of botulinum toxin, *i.e.*, smooth muscles paralyzed by the action of the toxin on their cholinergic nerves still react to acetylcholine.

Following the injection of exotoxins, a period of incubation elapses before symptoms of intoxication appear. This incubation period may be very short or may extend to 36 or 48 hours or longer. The usual incubation period of tetanus toxin is 36 hours, but it may be reduced to 35 to 60 minutes by the injection of very large amounts, 500,000 MLD, of crystalline toxin.

The discovery that the toxic qualities of the soluble toxins are destroyed by treatment with formaldehyde—which, at the same time, leaves the antigenic and antitoxincombining properties of the toxin unimpaired—has been of the greatest practical importance in immunization procedures. Toxin so treated is called toxoid. It might be supposed that this observation would throw some light on the nature of the toxicity of these substances, since formaldehyde is known to block amino groups. Analysis of the formaldehyde-protein derivatives indicates that the aldehyde combines with primary amino and primary amide groups but not with secondary amide or phenolic groups. Because of the effect of formaldehyde on toxicity and the results of detoxification with ketene and phenyl isocyanate, some workers have supposed that the free amino groups of the toxin molecule, such as the ϵ -amino group of lysine, are intimately associated with toxicity.

ENDOTOXINS39, 40, 82, 95, 98

The endotoxins contrast sharply with the exotoxins in that they apparently occur intracellularly in many gram-negative bacteria, especially the enteric bacilli, as lipidpolysaccharide-polypeptide complexes. They are heat-stable, i.e., to boiling, in neutral solution but destroyed by mild acid hydrolysis; are not digested by proteolytic enzymes; are of relatively low toxicity, with the mouse LD₅₀ little less than 0.1 mg.; are only partially neutralized by antiserum; and have substantially the same activity regardless of their origin. They appear to be structural components of the bacterial cell, represent the O antigen and occur, for the most part at least, in the cell wall of the bacteria.

The endotoxins may be extracted from the intact bacterial cell with trichloracetic acid or glycols or aqueous ethyl ether³⁴ and are present in solutions of the cell substance prepared by mechanical disruption either before or after digestion with proteolytic enzymes. The complete complex is extracted in trichloracetic acid and in glycol, and in this form the polypeptide portion does not give the usual qualitative tests for protein. This kind of preparation is known as a Boivin, or Boivin-type, antigen since it was first prepared by the trichloracetic acid extraction method devised by Boivin. The bulk of the lipid fraction may be removed by treatment of the complex with hot formamide and is apparently unrelated either to toxicity or antigenicity. On further degradation the toxicity is often lost, and the free polysaccharide differs according to the kind of bacterium from which the endotoxin was prepared and is responsible for its antigenic character.

The nature of the toxicity is not clear, but Goebel and his collaborators showed that the endotoxin complex of the Flexner dysentery bacillus gave a nontoxic polysaccharide and a toxic acidic protein on acid hydrolysis, and on alkaline alcohol hydrolysis broke down to a toxic polysaccharide and a nontoxic protein. The toxic protein could be detoxified with alkaline alcohol, and the toxic polysaccharide by acid hydrolysis, but it was not possible to characterize the toxic element. Subsequently, a method of extraction with 50 per cent phenol, and removal of the phenol by dialysis or extraction with an immiscible solvent, has been widely applied, 134, 135 which yields a lipopolysaccharide representing the endotoxicity. The small amount of contained lipid is distinct from the larger amount, 10 to 15 per cent, of the Boivin type of preparation and is not removed by extraction with appropriate solvents, and it is presumed to be an integral part of the toxicity. Such preparations may be taken to represent the toxic polysaccharides derived from Boivin antigen by various means. 125

On parenteral inoculation, the endotoxins produce a rise in body temperature, and are sometimes known as bacterial pyrogens, but their pharmacological activity is characteristic in the sense that they increase permeability.127 Capillary permeability may be increased with the production of local hemorrhage, and it is of interest that this occurs more readily in tumor tissue than in normal tissue so that if the dose is adjusted properly hemorrhage may be produced in tumor tissue only. The permeability effect is of interest also in that disease produced by many of the enteric bacilli is characterized by diarrhea. Following intracardial inoculation, grossly apparent edema and congestion with microscopic hemorrhages are produced in the small intestine within an hour or so.11 A characteristic inflammatory response is given on intradermal inoculation, commonly of the rabbit, which may be used for titration of the endotoxic activity61 by inoculation of endotoxin alone, or by potentiation with epinephrine.126 The reaction to endotoxin is strikingly similar to the Shwartzman phenomenon (see below), and it is believed by some that this may account for some, or even a major portion, of their toxicity.

ENDOTOXINS 269

The resemblance of severe intoxication to shock is striking, with fall in arterial blood pressure and rise in venous pressure due to pooling of blood in the portal system, and coincident development of leucopenia and thrombocytopenia. It differs from anaphylactic shock in that clotting time does not increase.132 Endotoxin shock may also occur in bacteremias due to gram-negative bacilli. As noted elsewhere (Chap. Seven), these infections have become much more common with wide use of antibiotic therapy, and such shock becomes of considerable significance because it is often very difficult to differentiate from shock due to myocardial infarction.133

A curious consequence of the parenteral inoculation of endotoxin is an increased resistance of the recipient animal to challenge infection with bacteria or viruses. 118 The mechanism of the effect is quite uncertain, but in the case of viral challenge may be related to the mobilization of endogenous interferon (Chap. Four).

Contrary to the usual impression, the endotoxins are excellent antigens and stimulate the formation of agglutinating and precipitating antibody to high titer. The antibody, however, fails to neutralize the toxicity to more than a minor extent; the endotoxins thus resemble in this respect some artificially prepared pharmacologically active azo antigens whose activity is not neutralized by antibody, though they combine with it.

Viral and rickettsial toxins. 18, 19 Toxic substances are associated with a number of viruses and rickettsiae which are endotoxins in the sense that they have not been dissociated from the infectious agent. While the pathological changes produced by these activities include effects on permeability, they are set apart from the bacterial endotoxins in that they are heat-labile and are neutralized specifically and more effectively, possibly in multiple proportions, by antiserum. Nothing is known as yet of their chemical nature.

Influenza virus, in doses of 1500 to 2000 hemagglutinating units, produces similar changes in the mouse on intravenous inoculation, the more toxic strains producing effects on the intestine such as edema and vascular engorgement. On intracerebral inoculation in the mouse, neurological signs are produced with tremor, clonic convulsions changing to tonic convulsions, and

meningo-encephalitic changes in the brain. Intravenous inoculation of mice with Newcastle disease virus produces changes similar to those found with influenza virus, e.g., lung consolidation and hemorrhagic enteritis.

Microorganisms of the psittacosis-lymphogranuloma group contain a labile toxicity demonstrable by intravenous inoculation of mice, but large doses, equivalent to 40 million infective doses, are required to kill. Mice dying early, *i.e.*, within 12 hours, show lung congestion with scattered hemorrhages, edema fluid in the alveoli, fibrin thrombi in the glomerular capillaries in the kidneys, and foci of damage or necrosis in the liver. In animals dying later, liver damage is the most pronounced feature with numerous necrotic foci accompanied by hemorrhage.

Of the rickettsiae, those causing the typhus fevers and scrub typhus, and possibly also spotted fever, form similar toxins. In the mouse and rat, engorgement of visceral blood vessels and hemorrhagic enteritis with accompanying edema are common features of the acute intoxication. More detailed studies have shown that, shortly after inoculation, vasoconstriction with leakage of plasma and altered capillary permeability occur in the mouse. ¹³¹ In addition, the toxin of the rickettsiae, of endemic typhus at least, produces extensive intravascular hemolysis in the rabbit. ⁸⁶

It is not clear to what extent viral and rickettsial toxins may contribute to the disease process. For example, in one cycle of influenza virus development, 40 to 60 infective particles are liberated from the infected allantoic cell. In volume these represent only a minute portion of the total volume of the cell, perhaps 1.5×10^{-5} , and yet it is sufficient to cause death. The implication would seem to be that replication involves some vital portion of the cell, for the postulated toxicity of the virus to produce death is improbably high. On the other hand, accumulated toxicity may well be a contributing factor in, for example, altering capillary permeability.

OTHER TOXIC SUBSTANCES

In addition to exotoxins and endotoxins, a variety of other substances are formed by microorganisms which may contribute to the disease process, either directly or by facilitating the establishment of a focus of

infection. Some of these are cytotoxic, affecting the red and white blood cells, others interfere, directly or indirectly, with the clotting mechanism, and still others are enzymes catalyzing the decomposition of structural elements of the tissues such as the hyaluronic acid ground substance and collagen. The more important of these activities may be considered briefly.

Hemolysins. A variety of bacteria produce hemolysins, substances which bring about the dissolution of the red blood cells of higher animals. The bacterial hemolysins, which are to be distinguished from the immune hemolysins formed by an animal in response to the injection of red cells of another species, are of two types, the so-called filterable hemolysins, which are extracellular and may be separated from the bacterial cells by filtration, and the hemolysins, which are demonstrated by the cultivation of bacteria on semisolid mediums containing whole blood.

Filterable hemolysins. The filterable hemolysins are sometimes named after the bacteria which form them; a streptolysin, for example, is a hemolysin produced by streptococci, and a staphylolysin a hemolysin of staphylococci. Hemolytic activity is demonstrated by the addition of filtrate or whole culture to a suspension of washed erythrocytes in physiological salt solution; after a period of incubation the red cells are laked, and hemoglobin appears free in solution. The relation of these hemolysins to other naturally occurring hemolysins such as saponins, the hemolysins present in snake venoms, and the like, is uncertain.90 The bacterial hemolysins appear to be protein in nature, are inactivated by heating (55° C. for 30 minutes), and are antigenic; i.e., when injected into animals they stimulate the formation of antihemolysins.

That hemolytic activity is a property of a group of substances of bacterial origin rather than of a single substance formed by a number of bacterial species is indicated not only by differences in immunological specificity but also by the varied properties of the activity. Many hemolysins, for example, are oxygen-stable, but some, produced by the pneumococcus, certain strains of streptococci, and some of the sporulating anaerobes, are oxygen-sensitive; i.e., they are active in the reduced form but inactive in

the oxidized form, the oxidation-reduction being reversible at low temperatures. Hemolysins further differ from one another in their heat and acid resistance and in the incubation time which precedes visible laking of the red cells. Differences in their activity on the erythrocytes of various species of higher animals may be marked; a given hemolysin, for instance, may lyse sheep cells but not rabbit cells. In general, the cells of the animal species from which a bacterium is isolated are more sensitive to its hemolysins than are the cells of other animal species.

A single bacterial strain may form more than one hemolysin. Certain strains of staphylococci form at least two hemolysins, one acting on both rabbit and sheep cells and bringing about a rapid lysis at 37° C., and the other acting only on sheep cells, which are lysed after holding at room temperature overnight—a phenomenon which has been termed "hot-cold" lysis. The former is termed α lysin and the latter β lysin. The action of both can be shown in culture on sheep blood agar, the α lysin producing a zone of complete clearing on 24 hours' incubation and the β lysin producing a zone of darkening which becomes lighter and

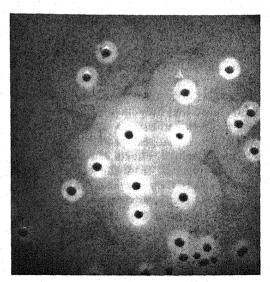


Figure 57. "Hot-cold" hemolysis by Staphylococcus aureus on sheep blood agar, produced by holding the plate alternately at incubator and refrigerator temperatures. The small clear zones are produced in the incubator by the α lysin and the large zones in the refrigerator by the β lysin.

HEMOLYSINS 271

clear on subsequent refrigeration of the plate. Staphylococci produce still other hemolysins in addition to these (Chap. Fifteen).

The occurrence of multiple hemolysins and hot-cold lysins is by no means limited to the staphylococci. Many streptococci, for example, produce two hemolysins, an oxygen-labile heat-stable hemolysin called streptolysin O and an acid-sensitive heat-labile streptolysin S. The occurrence of multiple hemolysins is especially marked among the sporulating obligate anaerobes making up the gas gangrene group. For instance, the most frequently occurring of these, Clostridium welchii, produces three toxins, the α , θ , and κ toxins, of which the first two are hemolysins; and the α toxin is a hot-cold lysin and a lecithinase.

The production of hemolysins is sometimes favored by inclusion of serum in the broth in which the microorganisms are cultured, and it is of some interest that the activity of the hemolysin formed in such mediums is most marked on the red cells of the animal species from which the serum was derived. The hemolytic activity of a culture may be transitory, probably because of inactivation of formed hemolysin. Some hemolysins lose their activity when incubated at 37° C. for two hours or more.

The precise manner in which the structure of the erythrocyte is altered so that contained hemoglobin is released is not clear, but it is an all-or-none phenomenon in that a single cell is not partially lysed. Like certain other hemolytic agents, the bacterial hemolysins apparently alter the surface of the cells, and swelling occurs prior to lysis. The staphylococcal α -hemolysin and streptolysin S have been found to lyse bacterial protoplasts as well as red cells.

Blood-plate hemolysis. The colonies of some bacterial species on blood agar produce visible changes in the medium immediately surrounding the colony which are termed "hemolysis." Two general types of change are observed, one designated as α or green hemolysis, in which the bacterial colony is surrounded by a zone of greenish discoloration, and the other, β hemolysis, in which the zone around the colony is clear and uncolored, in contrast with the red opacity of the medium. Microscopic ex-

amination of the green zone shows the presence of many discolored corpuscles, but in β -hemolytic zones corpuscles cannot be found

The relation between blood-plate hemolysis and the filterable hemolysins is usually regarded as uncertain, for microorganisms hemolytic on blood plates may not appear to produce filterable hemolysin; some workers maintain that, since filterable hemolysins are difficult to demonstrate, failure to find them is not significant. It would appear that the processes involved are different: in the case of the filterable hemolysins the permeability of the red cell is altered and the hemoglobin escapes into the surrounding fluid, while in blood-plate hemolysis the pigment is broken down to green or colorless compounds. In the case of the cholera vibrio the two processes have been sharply differentiated, since the identification of this organism is dependent in part on the hemolysis of goat erythrocytes in suspension (Greig test); though the true cholera vibrio is negative to this test, some strains show hemolysis, or hemodigestion, on blood agar plates (Chap. Twenty-three). On the other hand, there appears to be a close association between β hemolysis and streptoly-

The mechanism of β hemolysis is not well known, but in the case of staphylococcal hemolysins, the hemoglobin is not destroyed; according to Christie and Graydon the clearing around the colony is due to migration of the liberated hemoglobin. Green hemolysis was formerly thought to be due to the formation of methemoglobin and associated with the formation of hydrogen peroxide, but it has been found that the green substance is an iron-containing derivative of hemoglobin possibly formed by reduction. As in the case of the filterable hemolysins, hemolysis on blood agar is frequently species-specific; a higher proportion of bacterial strains will show hemolysis on blood from one animal species and not on that of another.

The relation of ability to form hemolysin and virulence is obscure. Although among some of the parasitic bacteria such as streptococci and staphylococci virulence is associated with hemolytic activity, a number of saprophytic bacteria also produce hemolysins. On the other hand, it has been

reported that in staphylococcus infections of the udder, the hemolytic strains are much more irritating than the nonhemolytic organisms. It might be expected that infection with actively hemolytic bacteria would be accompanied by anemia and hemoglobinuria, but this is not the case as a rule; infections with Cl. welchii, however, are frequently characterized by gross blood destruction and consequent severe anemia and jaundice which is due to the continuous release of the hemolytic α toxin. The massive intravascular hemolysis and associated hemoglobinuria, together with hyperkalemia, produced by the toxin of scrub typhus rickettsiae referred to above, may be a significant part of the pathological picture of this disease. In many instances, of course, a bacterial hemolysin may be toxic in other ways; i.e., bacterial toxins are sometimes hemolytic.

Leucocidins. A number of bacteria, nostaphylococci, streptococci. tably pneumococci, form leucocidins, substances which kill and, in some cases, lyse, polymorphonuclear leucocytes. The death of the leucocytes results in the inability of these cells to reduce methylene blue, a phenomenon made use of to demonstrate the activity of these substances.72 A direct microscopic method using phase contrast microscopy has shed some light on the effect of these substances on the white blood cell. The leucocidin so demonstrable is the socalled Panton-Valentine (PV) leucocidin; this leucocidin is a two component system in which the two factors, separable by ion exchange chromatography, function synergistically. Human and rabbit leucocytes in contact with leucocidin become spherical; the nuclei fragment and the cells may, with some leucocidins, eventually burst or, with others, remain intact.

The amount of leucocidin produced by one bacterial species may vary widely from one strain to another, and a single strain may produce more than one leucocidin. The detection of more than one leucocidin is dependent upon differences in thermostability and in the kind of leucocytes attacked; one staphylococcal leucocidin, for example, affects both rabbit and human leucocytes, while another is active only on rabbit cells. In general, the leucocidins closely resemble the filterable hemolysins in that they are antigenic, variable in their heat resistance,

and so on, and, in some cases, may be identical with hemolysins. The α hemolysin of staphylococcus, for example, is a leucocidin and is known as the Neisser-Wechsberg leucocidin. They may not be strain-specific; some of the staphylococcal leucocidins, for example, are immunologically similar if not identical regardless of the bacterial strain from which they are derived.

The part played by the leucocidins in the virulence of a bacterium is obscure. Theoretically, it should be of some advantage to an invading microorganism to be able to destroy these phagocytic cells and thereby break down, to some degree, the defenses of the body. The polymorphonuclear leucocytes are not, however, the most important cells in the phagocytic destruction of bacteria, and the effect of these substances on the other phagocytic cells, the mononuclears, the histiocytes, and others, is not well known. As in the case of the hemolysins, the leucocidins of bacterial origin must be differentiated from the immune leucocidins, those produced by the animal body in response to the injection of leucocytes from another species.

Coagulase. The formation of blood clots is accelerated by coagulase, a substance of bacterial origin. The production of coagulase is apparently confined to the staphylococci for the most part and may be causally related to thrombus formation that is common in infections with these organisms. Occasional strains of other bacteria such as Pseudomonas aeruginosa, Serratia marcescens, Escherichia coli, and Bacillus subtilis have also been observed to accelerate the clotting of blood. Coagulase activity is demonstrated by the addition of bacteria to citrated or oxalated blood plasma (human or rabbit), which gels within three hours. It is also demonstrable by mixing the bacteria with plasma on a slide and examining for the clumping that occurs with fibrin formation—the plasma-clumping test.

It has been found that the formation of a clot occurs in two stages, viz., a reaction between the substance produced by the bacteria, procoagulase, with a cofactor or activator present in plasma to form the clotting agent, coagulase, and clotting of the plasma under the influence of coagulase. While, then, strictly speaking, the bacterial factor is procoagulase, it is commonly referred to as coagulase. It is relatively heat-

stable and may be obtained cell-free with some difficulty. It is antigenic, although with some complications, and occurs as a number of antigenic types, more than one of which may be produced by a single strain of staphylococcus. The plasma factor is closely similar to, but not identical with, prothrombin from which it may be separated by Seitz filtration (prothrombin is retained) and is associated with the globulin fraction. There is some evidence that coagulase occurs in two forms, one bound to the cell and the other free; one bound to the cell and the other free; the former is presumably involved in the plasma-clumping test.

The distribution of the plasma factor among animal species accounts for the differences in the clotting behavior of plasma; most coagulases clot human, rabbit, and horse plasma, but not rat, chicken, or guinea pig plasma, though the last may clot slowly at 25° C. but not at 37° C. It is generally agreed that the formation of coagulase by staphylococci is closely associated with virulence, and reference is commonly made "coagulase-positive to staphylococci," implying virulent staphylococci. There is an association between the susceptibility of animals to staphylococcal infection and formation of the plasma cofactor. Thus rats, mice, and guinea pigs are usually relatively resistant to experimental infection with coagulase-positive staphylococci, and the virulence of coagulase-positive staphylococci for such animals can be enhanced by inoculating with bacteria suspended in coagulable plasma. Apparently bacteria coated with a film of fibrin are phagocytosed with difficulty, and, in addition, the clumping of both bacteria and phagocytic cells by fibrin tends to inhibit mechanically the process of phagocytosis. Further, it has been observed that coagulase antagonizes, to a greater or lesser extent, the antibacterial activity of normal serum, and coagulasepositive bacteria are able to grow in normal serum while coagulase-negative are not.28

The bacterial kinases. A number of kinds of bacteria, notably the streptococci, are able to dissolve fibrin clots or inhibit the clotting of plasma. This activity was described by Tillett and Garner in 1933 as fibrinolysin. It may be titrated as the highest dilution that will lyse a fibrin clot in a given time, or as the time required to lyse a fibrin clot, or, finally, by the amount of

protein made soluble in trichloracetic acid. The last is the least sensitive because dissolution of the clot occurs earlier than proteolysis to trichloracetic acid—soluble substances or free amino nitrogen.

The reaction does not occur with a clot formed by the interaction of purified human thrombin and fibrinogen. It has been shown that the factor produced by streptococci is not in itself the lytic substance, but a kinase that activates a precursor (plasminogen) of a plasma protease (plasmin) occurring in the euglobulin, and that the plasmin is actually the lytic agent. The bacteria activity, then, is not a fibrinolysin as it was originally and still is called, and is more properly referred to as a kinase, streptokinase in the case of the streptococcal activity.

Streptokinase. There are certain anomalies in the bacterial activity. For example, streptococci of human origin will lyse human plasma clots, but the plasma of most animals is not susceptible. Animal fibrin is, however, susceptible if clotted with human thrombin, and human fibrin is susceptible if clotted with animal thrombin. Moreover, rabbit clots become susceptible if mixed with plasminogen of human origin. Such observations are in large part explained by the occurrence of both free plasmin and a plasmin inhibitor, antiplasmin, in plasma, the latter in the albumin fraction. Thus the insusceptibility of rabbit plasma is due to the presence of relatively large amounts of antiplasmin rather than a deficiency in plasminogen as once thought. Similarly, on dilution of plasma, the plasmin-antiplasmin complex dissociates, and the solution becomes fibrinolytic. Inactivation of antiplasmin by chloroform has been utilized as the basis of a commercial purification of antiserum; the antiserum is shaken with chloroform and incubated to allow the preferential digestion of serum proteins other than antibody globulin to occur.

Fibrinolytic, or lysokinase, activity occurs among bacteria other than the streptococci. That of staphylococci, staphylokinase, is somewhat different from the streptokinases in species specificity and other respects; there is some evidence that staphylococci that produce β lysin (hemolysin) do not produce staphylokinase. A variety of other bacteria, including enteric bacilli and some of the sporulating obligate anaerobes, digest fibrin clots, but for the most part not a great

deal is known about these activities and possibly in some instances the digestion is due to proteolytic enzymes rather than kinases.

The streptokinases are antigenic and those from different strains appear to be very similar immunologically. Antiserums specifically inhibit the activity, and the occurrence of antikinase in the serum has been useful as an indicator of past infection with kinase-positive streptococci.

A number of bacteria exhibit a tendency to inhibit the clotting of plasma, a property that may be demonstrated by delaying the addition of calcium to the oxalated plasma; when calcium is added after a period of preliminary incubation, the clot fails to form. This property has been most often demonstrated with cultures in dextrose broth, and, in some cases at least, the failure of the plasma to clot is a result of the presence of lactic and other acids formed in the fermentation.

Streptokinase appears to be closely associated with virulence and particularly with the ability to invade the body tissues. Since one of the first reactions of the body to tissue destruction is the formation of blood clots which tend to wall off and isolate the infected region, it is not surprising that a bacterium capable of lysing these clots should show marked tendencies to extensive tissue invasion. The streptococci are among the most invasive of the pathogenic forms, and this phase of their virulence may be attributed in part to the formation of fibrinolysin.

Hyaluronidase (spreading factor, invasin).25,73 The permeability of the tissues is remarkably increased by a factor, often called the Duran-Reynals factor after its discoverer, present in certain mammalian tissues, notably the testes. Bacteria, vaccinia virus, and substances such as toxins, India ink, and the like, diffuse rapidly from the site of inoculation when injected in conjunction with extracts containing this factor. Strains of staphylococci and streptococci that have no pronounced invasive powers may, in this way, be rendered highly invasive, and hyaluronidase is apparently essential for producing inhalation infection.^{21, 55} This factor is also present in a number of bacteria notable for their invasive properties, such as certain strains of staphylococci and streptococci, pneumococci, and certain of the obligate anaerobes such as the bacillus of gas gangrene. Among some of these organisms there is some degree of association between content of the Duran-Reynals factor and virulence; the noninvasive strains of staphylococci noted above contain little or none of this factor, but become invasive when it is supplied, while the invasive strains produce the factor themselves.

This substance is an enzyme, hyaluronidase, whose substrate is hyaluronic acid, a mucopolysaccharide consisting of acetylglucosamine and glucuronic acid, which acts as a cement substance of the tissues and is found in synovial fluid and elsewhere in the body.

Decomposition of hyaluronic acid occurs in three stages, viz., in the first it is no longer precipitable with acetic acid, the second stage is that of depolymerization and coincident decrease in viscosity, and the third is characterized by the liberation of reducing sugars. Each of these has been used for assay of hyaluronidase activity, and most in vitro methods are based on viscosity changes. The action of the enzyme in decreasing the viscosity of hyaluronic acid by hydrolysis is responsible for facilitating the penetration of the tissues by bacteria that produce it. The effect on the tissues is temporary in that, following inoculation of preparations of the enzyme, the dermal barrier is restored partially in 24 hours and completely in 48 hours.

It is of some interest that hyaluronic acid is also found in the capsular substance of some strains of streptococci. Such strains do not produce hyaluronidase and in its presence are denuded of capsules and more readily phagocytosed (see below). The ability of such strains to invade the tissues does not, of course, depend upon their elaboration of hyaluronidase. The activity appears to be antigenic, in that it is neutralized by antiserums, but immunologically distinct from different sources; it has been suggested that the enzyme is combined with different proteins in different organisms.

Hyaluronidase is antagonized by an enzyme present in normal blood plasma which has been called anti-invasin I. An enzyme in bacteria, pro-invasin I, destroys anti-invasin I, and another enzyme in plasma, anti-invasin II, destroys pro-invasin I. It has been suggested that a balance between

CAPSULES 275

all these in the host-bacterial system determines whether or not invasion of the tissues will occur.

Streptodornase. Streptodornase is a deoxyribonuclease produced by many hemolytic streptococci, together with streptokinase, which depolymerizes viscous DNA, and its activity may be titrated by viscosity methods. It appears to be unrelated to virulence. Both streptodornase and streptokinase are available for the enzymatic debridement of wounds. The functioning of the latter depends upon the presence of sufficient plasminogen. Plasminogen is not available generally for this purpose, but may be prepared from human plasma.¹⁷

CAPSULES

The association between the presence of a capsule on a pathogenic bacterium and its virulence has been discussed elsewhere (Chaps. Three and Seven). The capsular material, generally polysaccharide in nature although nitrogen and amino acids may be present, is not in itself toxic and cannot be regarded as analogous to toxins, hemolysins, and similar substances. Rather the capsule appears to function as a defensive mechanism of the bacterium against the phagocytic activity of the leucocytes. Encapsulated bacteria may be ingested by a white blood cell but, instead of being killed and digested. remain within the phagocyte for a time and then may be extruded in a viable condition. The ability of an encapsulated bacterium to resist phagocytic destruction may, in fact, result in a wider distribution of the microorganism than it might otherwise attain, through transport within the phagocytic cell. It is of some interest in this connection that nonencapsulated, avirulent pneumococci are highly virulent for rabbits deprived of their leucocytes. It is perhaps suggestive that the polypeptide material making up the capsule of the anthrax bacillus contains D(-) glutamic acid. Since proteolytic enzymes attack only polypeptides built up from amino acids of the L-series, possibly an encapsulated anthrax bacillus would be highly resistant to the digestive enzymes of a phagocytic cell.

Whether the association of virulence and capsule formation may be entirely accounted for on the basis of bacterial defense against

phagocytic destruction is not entirely certain, but such defense undoubtedly plays an important part. The presence of antibodies to the capsular substance breaks down this bacterial resistance, and immunization to the capsular material of the pneumococcus, for example, produces just as high a degree of immunity to pneumococcal infection as immunization to the entire bacterium.

MISCELLANEOUS FACTORS

In addition to these more or less well defined and better known factors, a number of bacteria have been reported to produce substances which may be associated with virulence. Such, for example, are the necrotizing factor, or necrotoxin, produced by some staphylococci, which kills tissue cells; a hypothermic factor produced by Shiga dysentery bacilli, which lowers body temperature; an edema-producing substance formed by pneumococci; substances associated with the endotoxins of some of the enteric bacteria which affect the blood sugar levels of animals. Unfortunately, the discontinuous character of present information does not support any satisfactory generalization regarding bacterial virulence, but it seems clear that a pathogenic bacterium may have at its disposal a series of mechanisms, in combination peculiar to itself, which make possible a successful invasion of the tissues of the host.

TOXICITY OF HOST ORIGIN

It is not uncommon to observe signs of toxemia in infectious disease when the causative microorganism cannot be shown to form toxic substances in vitro. The classic example of this is the marked toxemia of acute pneumococcal infection, while the pneumococcus cell substance and metabolic products formed in culture are comparatively bland. Observations of this kind suggest either that toxicity is of host origin, in that it is produced by the infected host tissue, or that the microorganism produces toxic substances in the environment of the infected tissue but not in culture in vitro.

There are two obvious alternatives in the former. It may be postulated that, in the interaction of the metabolic systems of the proliferating microorganism and the host cells, those of the latter are perverted with the accumulation of substances toxic to the host. While there is no definitive evidence of such interaction, the plausibility of the hypothesis is enhanced by the relatively rapid disappearance of signs of toxemia on institution of chemotherapy. The chemotherapeutic drugs are not antitoxic, though some small effects on microbial toxins have been described, but they inhibit proliferation of the microorganism, suggesting that the active metabolism of the growing microorganism may be associated with toxicity. Perhaps similarly, in gaseous gangrene (Chap. Twenty-nine) the host is in shock although systemic toxin is not detectable, and antitoxin is ineffective; amputation of the affected limb relieves general symptoms.

The other alternative is a more or less specific interference by a bacterial product with a functional system of the host. Two examples of this have been described above. One is the activation of plasminogen by the antagonism of the bacterial kinases, such as streptokinase, for its normal inhibitor, antiplasmin. The other is the staphyloinaccurately product, coagulase, which activates a plasma cofactor separable from prothrombin. In some instances it may be shown that toxic substances are produced by the host tissue. The thromboplastic toxin, or necrosin, that may be isolated from skin lesions of streptococcal etiology may also be found in normal skin subjected to traumatic iniurv. 20, 103

Other phenomena of this kind are less well understood. There is, for example, good evidence that the febrile response to bacterial substance often is not due to pyrogenic substances present in the microorganism but is a consequence of an interaction between the bacterial substance and plasma to form, or liberate, pyrogenic substances of endogenous origin.43 Still another such phenomenon is the greatly increased sensitivity to histamine, on the order of a hundred-fold, that is developed following inoculation with pertussis bacilli and that is perhaps attributable to an effect on the rate of histamine release from the tissues. This kind of "hypersensitivity" is possibly related to the pathogenesis of whooping cough in which the "whoop" persists after

the bacteria can no longer be found in the infected individual. In this connection it is suggestive that the sensitivity to histamine so produced is more marked in female than in male mice, and in whooping cough the general rule that mortality from infectious disease is significantly greater in the male is reversed.

The Shwartzman phenomenon. 108, 128 The reaction known as the Shwartzman phenomenon may be regarded as a special case of a host reaction resulting in increased sensitivity to microbial substances. It occurs most readily in the rabbit and is produced by two inoculations, a "preparatory" inoculation and a "provocative" inoculation, a few (8 to 30) hours apart, and the reaction so produced may be local or general. In the former, the preparatory inoculation is given intradermally, and the provocative inoculation intravenously; a short time after the second inoculation gross hemorrhage and necrosis develop at the site of the preparatory inoculation. The general reaction is induced by giving both inoculations intravenously and is characterized by bilateral cortical necrosis of the kidneys. While the time element and the lack of necessity for a common antigenicity in the preand provocative substances paratory precludes an immunological basis for the reaction, it resembles overtly the Arthus phenomenon (Chap. Fourteen) and is intensified when the animal has received immunizing inoculations of the preparatory material.

Not all bacteria contain preparatory substances, but the endotoxins of the enteric bacilli are almost uniformly effective. It has, in fact, been suggested, especially by Stetson, that the toxicity of many bacterial endotoxins may be a manifestation of the Shwartzman reaction, possibly enhanced by an immune response in some instances, in which the preparatory dose is absorbed from the bowel as endotoxin of coliform and related components of the normal intestinal flora. There is a wider range of provocative than of preparatory substances, and substances such as starch or agar, serum, or bacteria which have no preparatory effect may be provocative.

The participation of the host in this reaction is indicated in a number of ways. Following the preparatory intradermal inoculation, there is a local cellular reac-

RESISTANCE 277

tion around the venules in the area, an increased glycolysis associated with the influx of heterophils, and a local accumulation of lactic acid. This response, and subsequent reaction to the provocative inoculation, is prevented by treatment which induces leucopenia, such as x-irradiation or the administration of nitrogen mustards. The significance of the cellular response is also indicated by the enhancement of the reaction when the macrophage system is blockaded with Thorotrast and an increased sensitivity to endotoxin preparatory inoculation when the animal is treated with cortisone. In total, the evidence suggests the preparatory inoculation interferes with a significant detoxifying mechanism.

Local infection with a variety of bacteria, such as tubercle, anthrax, and influenza bacilli and streptococci, are preparatory even though the microorganism itself may not be preparatory. Thus streptococci are not preparatory though a local streptococcal infection may be, and preparatory activity may be demonstrated in extracts of the infected tissue. The extent to which the Shwartzman type of reaction contributes to the pathogenesis of infectious diseases is, however, not at all clear.

Microbial toxins formed in tissues. The second possibility alluded to above is that of the production of toxin by the microorganism only in the environment provided by the infected tissue. This is suggested by, for example, the increased virulence that may be induced by animal passage, the inability to differentiate *in vitro* between virulent and avirulent strains of certain microorganisms such as the plague bacillus, and by the contrast between anthrax bacilli *in vitro* and *in vivo*.

The last is one of the most thoroughly studied instances of this kind. It is considered in more detail elsewhere (Chap. Twenty-eight) but may be noted briefly here for illustrative purposes. There is little or no evidence of toxicity of the usual in vitro culture of the anthrax bacillus, nor can effective killed vaccines be prepared from such cultures even though the bacilli may be shown, by animal inoculation, to be highly virulent and to produce an immunizing infection. While a number of factors are involved, it suffices for present purposes to note that an effective immunizing antigen may be extracted from infected tissue, and elucidation of the nutritive requirements for its production have allowed the development of culture mediums in which it is formed in vitro. This immunizing substance is not of sufficient toxicity to account for the death of infected animals. but such toxicity is demonstrable in the plasma of infected animals. This toxicity is separable into two fractions, one of which is sedimented by high speed centrifugation while the other is not. Separately the two are only moderately toxic, but when combined their toxicity is synergistic. The sedimentable fraction resembles the immunizing antigen, differing in that it is toxic when combined with the nonsedimentable fraction, and the immunizing antigen may be a "toxoided" form of the toxic fraction. It is not yet known in what way the tissue environment makes possible the formation of these toxic substances, but it is clear that the toxigenicity of these bacteria, and no doubt others also, in the infected animal is not adequately reflected in the toxic activities of in vitro cultures.

Resistance 106, 122

It is already apparent from the foregoing considerations of microbial virulence that, although the ability of a microorganism to produce disease is conditioned by a series of mechanisms originating with the microorganism, pathogenicity must be evaluated in terms of the resistance of the host. As a rule, a pathogenic microorganism is limited to a small number of hosts; microorganisms pathogenic for animals are not ordinarily

pathogenic for plants; very few of the microorganisms that can infect mammals are also pathogenic for cold-blooded animals; some are even restricted to the tissues of a single species. Resistance, like virulence, is made up of many factors, some of which are known either in more or less specific form or in the terms of generalities that serve as a cloak for ignorance; others are, in all probability, as yet unsuspected.

Resistance to infection is, in a sense, somewhat more complex than virulence for, as will appear, not only are there specific barriers to infection variable with respect to species and even from one tissue to another in the body of a single animal, but the efficiency of these barriers is also a manifestation of general physiological well-being, and hence they are subject to extrinsic or environmental stress.

The factors operable in the host are of two general kinds, the constitutive group, which includes those which occur in the normal animal, and the adaptive group which are the responses to the presence of the pathogenic microorganism. The latter are predominantly, though not entirely, those associated with the immune response, and are considered elsewhere (Chaps. Thirteen and Fourteen), while the former are discussed here.

Species. racial, and inherited resistance. $^{41, 123}$ Species of higher organisms differ greatly from one another in their resistance to any given disease, a fact that has been suggested earlier in connection with the experimental reproduction of disease. In many cases resistance to infection is relative, for disease may sometimes be produced by the administration of massive doses of bacteria to a resistant animal, but in others it appears to be absolute. Man is. for example, apparently completely immune to cattle plague, and many of the lower animals are equally resistant to some diseases of man. In general the factors underlying differences in species resistance are unknown, but in a few cases body temperature or differences in anatomical structure have been found to account for the observed variation. Pasteur's classic experiment in which he rendered the naturally resistant hen susceptible to anthrax by chilling it in cold water, and the converse of this experiment, the production of anthrax in the resistant frog by raising its body temperature from 25° to 35° C., may be explained in terms of unfavorable body temperatures. Similarly, cold-blooded animals are not susceptible to human tuberculosis, and warm-blooded animals are not infected by the tubercle bacilli of cold-blooded animals. 99 The insusceptibility of experimental animals such as guinea pigs and rabbits to the enterotoxin produced by some bacteria is possibly attributable to their lack of a vomiting mechanism. As indicated earlier, resistance to a given infectious agent is not necessarily associated with phylogenetic relationships, and there is no pattern from which the resistance or susceptibility of an animal can be predicted by logical processes; the tabulation of animals that are susceptible to a given disease represents information that has been acquired largely by trial and error.

Domestic and experimental animals. Not only do species of higher organisms differ in their resistance to infectious disease but the races comprising a susceptible species likewise appear to differ among themselves. There are many instances of differences in resistance to infectious disease in varieties or strains of animals. The relative resistance of Algerian sheep to anthrax is well known, and inbred Berkshire swine have been found to be highly resistant to brucellosis. That this is true racial immunity which is, as might be expected, inheritable has been demonstrated by extensive experimental investigations with laboratory animals. The earlier studies of Wright and Lewis showed that marked differences in susceptibility to tuberculosis existed between inbred families of guinea pigs, differences which were transmitted to the offspring. In subsequent work⁴² it has been shown that resistance to infection may be raised or lowered by selective breeding, sometimes to a remarkable degree. Lurie has bred strains of rabbits resistant and susceptible to infection with tubercle bacilli. When subject to inhalation infection with human tubercle bacilli, the response of the animal is essentially all or none and sharply illustrates the role of genetic factors; in the resistant animal the bacilli multiply for only a short time and are then destroyed, while in the susceptible animal they proliferate freely for long periods. 66 The resistance of mice to infection with tubercle bacilli seems also to be affected by genetic constitution.68

It is of interest that resistance to bacterial endotoxin may also be raised or lowered by selective breeding. Resistance does not behave as a simple Mendelian character but is to some extent specific in that a race having increased resistance to infection with one microorganism is not necessarily unusually resistant to another. For example, in Webster's work mice selected for resistance to infection with Salmonella enteritidis

showed increased resistance to pneumococcus and Friedländer's bacillus infection but were more susceptible to the virus of louping ill than the strain selected for susceptibility. In a general way, it appears that inherited resistance is, to a degree, specific in that it is effective against groups of related infections rather than against infection in general.

Considerable interest has attached to the mechanisms underlying inherited resistance and susceptibility. Thus, in Lurie's studies resistance was associated with low skin permeability as assayed by intradermal inoculation of India ink, increased rate and intensity of antibody (agglutinin) response, and the development of a high degree of hypersensitivity. It has been found that in mice there is a marked correlation between numbers of leucocytes and resistance to mouse typhoid, and macrophage function in general appears to be involved.65 Similarly, resistance and susceptibility of breeds of chickens to Sal. pullorum infection are associated with numbers of lymphocytes; differences in liver and spleen in resistant strains of mice are associated with the ability of macrophages to digest phagocytosed bacteria, and strains of chickens genetically resistant to Sal. gallinarum infection have higher body temperatures than susceptible strains, and this was combined with more active phagocytosis. Resistance to virus infections is also determined by genetic factors.1

While such correlations as the foregoing are suggestive, in general it has not been possible to associate resistance with any single character; rather it appears to be dependent upon a complex interaction of characters that, individually, do not account for the observed resistance. The inheritance of resistance to influenza virus, however, is considered to be determined by a single dominant autosomal allele.64 Differences in susceptibility may, of course, be a reflection of corresponding differences in immunizability, i.e., ability to respond to antigenic stimuli with antibody production, as in Lurie's resistant rabbits, and the immune response to toxoids behaves similarly.53

Races of man.¹⁰⁴ The relative resistance of races of man to infection has been the subject of considerable interest and such investigation as has been possible. Under

ordinary circumstances in this country the nonwhite races are much more susceptible to infectious disease than the white race. There are, however, certain exceptions. Thus, the influenza epidemic of 1918 appears to have had a greater impact upon the death rate for white youths than upon that for the nonwhite population in the same age group. A similar exception may be noted in the case of Baltimore Negroes, who showed a lower ratio of clinical diphtheria to immunizing infections than corresponding white children. It is quite generally recognized, too, that the Negro has a remarkable degree of resistance to erysipelas, and the more favorable response of the Negro to all forms of treatment for gonorrhea is well known.

Special interest has attached to the white and nonwhite tuberculosis death rates, both crude and age-specific. Whether the observed high mortality in the nonwhite represents a racial susceptibility or is entirely a reflection of economic status has been the subject of considerable discussion.⁵⁹

There also appear to be differences between the less well-defined "races" of man. There is evidence that the Irish are less resistant to tuberculosis than certain other elements of the American population, such as the Italians. On the other hand, the Jewish race is considered by many to be relatively resistant to tuberculosis; in spite of a high incidence of infection, the mortality is very low. To what degree the evidence supports the hypothesis that races of man differ in their susceptibility to this and other diseases, such as pneumonia, is problematical, for adequate control of the environmental factors is difficult if not impossible.

In general, appropriate data on the hereditary transmission of susceptibility, or resistance, to infection in man are not available, but two kinds of evidence suggest that hereditary factors are involved. The best of such data are twin studies, monozygotic and dizygotic, the latter serving as controls. Many of these have concerned tuberculosis, and most are in agreement that there is a hereditary element in susceptibility,110 and similar studies of susceptibility to poliomyelitis have indicated that susceptibility to the paralytic form of the disease may be conditioned by the heterozygous state of a recessive gene. 50 A hereditary element is suspected when a disease seems to "run in families." Rheumatic fever is such a disease, and the observed associations between incidence of the disease and blood relationship appear to be significant. But, in general, association of infectious disease—incidence or severity—with human blood groups is questionable. The question is one of the occurrence of practically significant genetic segregation.

One of the best established cases of hereditary control of resistance to a specific infection is that of the relative insusceptibility to certain kinds of malarial infection associated with sickle-cell anemia and the anemia, thalassemia.111 Mediterranean These anemias are almost invariably fatal in the homozygous individual, but the individual, heterozygous for the appropriate gene, is considerably more resistant to malarial infection than the individual in whom these genes do not occur. In sicklecell anemia hemoglobin synthesis is affected directly by the gene, and in the abnormal hemoglobin (hemoglobin S) valine is substituted for glutamic acid at one point in the peptide chain. In thalassemia the expression of the genes responsible for hemoglobin synthesis is affected quantitatively to give excessive production of the fetal type of hemoglobin (hemoglobin F). It is probable that the presence of such hemoglobins in the red blood cell makes it less susceptible to infection with malarial parasites.

A given disease, however, may be relatively mild in its effect on races of man that have been in contact with it over a long period, but may assume a highly virulent form in other races to which it is new. Measles, for example, a mild disease to civilized man, has been a scourge to certain primitive races. In other cases, diseases originally highly virulent have become apparently less so with the passage of time; leprosy is not as widespread as it was in Biblical times, and syphilis is a considerably milder disease today than it was in the sixteenth century. Phenomena such as these have been taken by some to indicate the development of a racial immunity through a selection of more resistant individuals, and by others to suggest an adaptation of the microorganism accompanied by a loss of virulence. At the present time, it is not possible to differentiate sharply between these two; possibly both effects are operative.

It should be pointed out in this connection that what might be called a pseudo-racial immunity may be manifested by a race in close association with a given infective agent. Many individuals have the disease. the survivors are immune, and this immunity is passively transferred to the offspring, who are infected before this passive immunity entirely disappears and consequently have the disease in a mild form but become solidly immune. The immunity is passively transferred to the third generation, and the process continues ad infinitum as long as the race is in contact with the disease. Some such mechanism as this appears to account for the apparent racial immunity of the West African Negro to yellow fever; the immunity of adults arises as a result of mild infection in childhood.

Age. 116 In a general way, resistance to infection increases with age, but there are a number of important exceptions. Embryonic and fetal tissues have little resistance, and susceptibility remains high for a variable, but short, time post partum. The chick embryo and associated membranes may be readily infected with a variety of agents, particularly viruses and rickettsiae, to which the chicken, is for all practical purposes, completely resistant. Similarly, on growth on culture in vitro, tissue explants tend to assume the physiological character of embryonic or neoplastic tissue and are susceptible to infection with a variety of agents. The persistence of susceptibility post partum is taken advantage of in the propagation of certain infectious agents, such as certain strains of poliovirus in suckling mice or the enteric infection of the suckling rabbit with the cholera vibrio.

An important factor, though not the only one, in the susceptibility of the very young to infection is the lack of an immune response. This is compensated for in the newborn mammal, so far as survival is concerned, by the presence of antibody of maternal origin (Chap. Fourteen) which serves to provide temporary protection against the diversity of microorganisms encountered on emerging from a sterile environment. Occasionally this may not be sufficient, and fatal systemic infection with microorganisms not usually regarded as pathogenic can occur. Such infections with coliform bacilli are occasionally observed in human infants, and the disease of

stood.

foals known as scours is a septicemia of coliform etiology. Apparently the immune mechanism is poorly developed even at birth, and the extremely young tissue is unable to distinguish the "not self" of foreign proteins and other antigenic substances. This has the practical consequence that prophylactic immunization of the newborn human infant is relatively ineffective.

The predominance of the diseases of childhood is not necessarily due to an unusual lack of nonspecific resistance in the pre-adolescent age groups. Man appears to be highly susceptible to diseases such as measles, mumps, and chickenpox, but the infectious agents are sufficiently prevalent in the human population that the probability of encountering them early in life is great. When the child is relatively isolated, as in certain rural areas, the probability of contracting such a disease is less, and it tends to appear later in life. It is for this reason that the so-called diseases of childhood tend to be a problem when young adults are suddenly aggregated as in military groups; such groups include a fair proportion of individuals who have, by chance, not vet contracted immunizing infections in childhood.

By puberty the average individual has acquired a variety of immunities, with or without overt evidence of infection, which are similar to the pseudo-racial immunity described above. The juxtaposition of such acquired immunities with puberty has led, from time to time, to the postulation of a "maturation immunity," a state of enhanced resistance presumed to be causally related to sexual maturity. Such an explanation for the greater resistance to diphtheria of the young adult was offered many years ago, but it was shown that this is, in fact, the result of immunizing, but inapparent, infections in childhood. Such an explanation of resistance to poliomyelitis on the part of adults was current for a time until it became clear that infection with poliovirus is very common, only the rare individual showing paralytic symptoms. Further, attempts to demonstrate a maturation immunity in experimental animals in which sexual precocity has been induced by the administration of sex hormones have failed to provide supporting evidence. The naturally occurring association between sexual maturation and resistance to infectious disease appears

to be fortuitous and not indicative of a causal relationship.

Other differences in susceptibility to infectious disease associated with age do, however, appear to have a physiological rather than an immunological basis. The two best established examples are those of the occurrence of acute fatal pulmonary tuberculosis in the young adult and the marked susceptibility of the aged to pneumococcus and similar pneumonias.

Infection with the tubercle bacillus (Chap. Thirty-one) is markedly influenced by predisposing factors, a characteristic of a wellestablished balance between host and parasite. In addition to factors which are grouped under the general head of stress, there appears to be a physiological susceptibility that begins to be evident in the teens and does not decline until the late twenties. This is reflected in the age distribution of death rates from this disease which, disregarding the high rate in the first year of life attributable largely to risk, reaches a peak in these age groups. The secondary peak of so-called late adult tuberculosis at around age 50 that has become apparent in the last three decades is another matter which is not relevant here. The physiological basis of heightened susceptibility to this disease of the young adult is not under-

The greater susceptibility of the aged to pneumonias is generally regarded as an expression of an accumulation of degenerative changes which find one expression in this way. It has not been possible to associate this susceptibility with specific physiological changes, but failure to accumulate immunities to microorganisms such as pneumococcus and streptococcus during the life history of the individual, because these immunities are transient, is no doubt a factor, but hardly a differential one.

Study of the effects of age on the susceptibility of experimental animals to tubercle bacillus³⁰ and streptococcal¹³⁶ infections has demonstrated significant effects of age; the effect on the susceptibility of the guinea pig to tuberculous infection appears to be similar to that in man. Age is a factor also in the susceptibility of mice to Coxsackie⁶³ and variola⁷⁰ viruses; and in experimental herpes infections the macrophage appears to be an important factor.⁵⁶

A consequence of the differences in re-

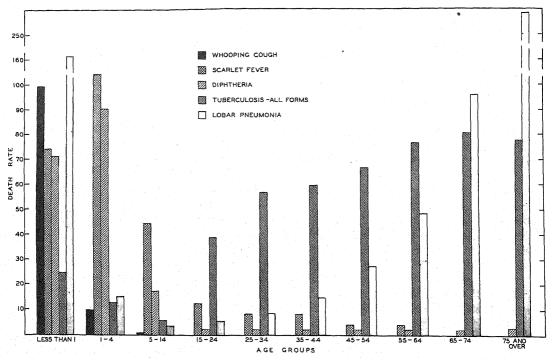


Figure 58. The age incidence of certain infectious diseases. Note the predominance of the diseases of childhood in the early years, the increase in tuberculosis in the young adult years, and the marked increase in lobar pneumonia with advancing age. Scarlet fever rate multiplied by a factor of 40 and diphtheria rate by a factor of 10 for comparative purposes. (Data from the reports of the Bureau of Census.)

sistance, or acquired immunity, to various pathogenic microorganisms associated with age groups is that the prevalence of disease, but not necessarily infection, is a function of the age distribution of the population (Chap. Ten).

Sex. A number of diseases exhibit a difference in sex incidence; pneumonia and epidemic meningitis, for example, are more common in males than females, while scarlet fever, typhoid fever, and others occur somewhat more frequently in females. Except for a few years in the 41-year period 1900-1940, the death rate for white female youths has been consistently lower than that for males, though the reverse has been true for the nonwhite population in this country. In the decade 1931–1940 the gap between the female and male in the nonwhite population was closed, and in 1940 the female and male death rates for nonwhite youths were exactly the same, at 5.0 per 1000. Corresponding rates for the white population were 1.4 for females and 2.0 for males. In the case of tuberculosis, variability in the agespecific mortality rates indicates a differential response on the part of the sexes. A sex differential is also indicated by the aftermath of the 1918 influenza epidemic, which appears to have had a more unfavorable effect upon female mortality than that of males in the 15- to 24-year age group, as indicated by the relatively high female death rate for all racial groups for several years after the epidemic.

It has been suggested that this difference between the sexes might result from resistance having some of the attributes of a sex-linked character, or, since differences in sex incidence are most pronounced during and after puberty, something in the nature of maturation immunity may be involved. There is no sound evidence to support the first of these hypotheses. Regarding the second, a certain amount of experimental evidence has been reported, the most precise relating to skin permeability, which has been found to be greater in female rabbits than in males. It has also been shown that female chicks are significantly more susceptible to infection with the chicken malaria parasite, *Plasmodium gallinaceum*,

than are males; treatment with male and female sex hormones did not affect the differences between the two. Any explanation of the observed sex differences must, of course, take into consideration other factors such as occupation, risk of exposure, and the like.

Climate and season.88, 119 That both climate and the season of the year exert marked effects on the incidence of and mortality in a number of infectious diseases is well known. In tropical climates, for example, the acute upper respiratory infections are not so common as in the temperate climates, but the dysenteries are more common in the tropics than in the temperate zones. The seasonal incidence of the infectious diseases is also common knowledge; meningococcus meningitis occurs predominantly in the winter and spring, poliomyelitis in the late summer and early fall, and so on. One of the most striking relationships of season to incidence of disease is that of cholera: the epidemic season coincides with hot weather and shows a remarkable correlation with precipitation and relative humidity. It is said that in the tropics, diphtheria is a rare disease and scarlet fever seldom occurs. Yet studies on the age incidence of positive Schick and Dick tests, indicative of immunity to these diseases, have shown that inapparent immunizing infections are quite as common as in temperate climates.31

In a great many instances the influence of climate and season upon the incidence of disease may be attributed to opportunities for transmission of the causative organism, such as crowding in poorly ventilated rooms, seasonal or geographic occurrence of an insect vector, and so on, but in others the factors responsible for seasonal incidence are unknown.

There is a good deal of evidence which substantiates the opinion that resistance varies with season, temperature, and similar factors. For example, the intensity of the brain reaction of mice inoculated with St. Louis encephalitis virus and of guinea pigs inoculated with endemic typhus rickettsiae has been found to be the greatest in summer and the least in the winter over a period of years; mice adapted to existence in a moist heat have been found only one-quarter as resistant to infection with hemolytic streptococci as those adapted to a cool environ-

ment; the morbidity and mortality rates in murine typhus infection in mice are affected by environmental temperature; and the resistance of mice to pneumococcus infection is similarly affected. On the whole, chilling reduces resistance to infection in experimental animals. 10, 57, 91, 129 In at least some instances seasonal variation in resistance may be correlated with diet (see below). It is not improbable that future investigation will yield systematic knowledge of the effect of climatic factors on susceptibility to disease.

General physiological well-being. Whatever resistance to disease an organism may possess by virtue of species, race, and the like, is profoundly influenced by its general physiological state. In general, resistance is at its height when the organism is functioning normally in every respect and is reduced by a variety of factors which interfere with and alter the normal physiological state. In some cases previous infection may reduce resistance to such a point that infection with the less virulent bacteria may take place, as in secondary infections; in others, functional disorders such as diabetes bring about a reduction in resistance to infection.9 More common, however, are the deleterious effects of inadequate diet and fatigue.

Nutrition.24, 101 The relation between susceptibility to infection and faulty nutrition has been of considerable interest in connection with the study of deficiency diseases. There is little doubt that nutritional status is of practical significance to the incidence of and mortality in infectious disease. The general problem has not responded to experimental study as readily as might be supposed. In a great many instances inanition is not clearly separable from a specific deficiency, such as a vitamin or amino acid deficiency, and results are complicated by such "side reactions" as dehydration resulting from reduced water intake by animals on certain deficient diets. In general, however, it seems to be clear that diets qualitatively and quantitatively inadequate may predispose to bacterial infection. The reduced resistance appears to be associated with a reduced humoral immune response, but this is in part at least compensated for by an accentuated phagocytic response to infection.

The importance of adequate protein intake has been emphasized by Cannon and his co-

workers, ¹⁴ and they have shown that depletion of the protein reserves of experimental animals by maintenance on low protein diets interferes markedly with antibody formation, *i.e.*, the synthesis of immune globulin. While antibody formation is significantly interfered with by moderate protein depletion, a severe depletion also interferes with the normal functioning of the cellular defense mechanisms as evidenced by reduced phagocytosis.

Other than this, just how malnutrition brings about a reduction in resistance is not known; present information indicates that the effect is a general one. It is of some interest that this unfavorable effect is not limited to the undernourished individual but may be transmitted to offspring. Experimental studies have shown, for example, that the diet of mice, genetically homogenous in resistance to an infection, affects the resistance of offspring to a greater degree than the diet of the offspring themselves. In this connection, statistical studies have suggested that the mortality rate during adult human life are in part a function of nutritive and other elements of the environment of childhood; in other words, the effect may be a delayed one, but the evidence on this point is not altogether indubitable. 16

The marked reductions in resistance associated with inadequate diets are in no sense specific; resistance to infection in general is reduced, and there is no relation between lack of a single dietary factor and susceptibility to a particular infection. A number of attempts have been made to demonstrate such a relation; ascorbic acid, for instance, has some small capacity to neutralize diphtheria toxin, but vitamin C deficiency does not predispose to infection with the diphtheria bacillus any more than with other organisms, such as the tubercle bacillus.

The apparently contradictory observation that resistance to infection with a number of the viruses is increased by inadequate diet has been made by a number of workers. That it is not completely inconsistent with the lowered resistance associated with protein depletion and other dietary deficiencies is indicated by the consideration that the viruses are obligate intracellular parasites; possibly a healthy cell is of primary importance to multiplication of the virus and the depleted cell is an unfavorable medium for development, thus masking, so to speak, the

lessened immune response associated with nutritional deficiencies.

More specific evidence in this connection has been reported for certain bacterial infections. It has been pointed out elsewhere that selection of virulent variants of a challenge microorganism may occur in vivo: virulent threonine-requiring variants of Sal. typhimurium are so selected by the animal given additional threonine.84 It has been found also that an induced variant of Sal. typhi having complex nutritive requirements showed at the same time reduced virulence for mice. The supposition that the animal did not supply the required nutrients in adequate amount was substantiated by the observation that full virulence was restored when the animals were given supplemental amounts of the nutrients required by the bacteria - purine, p-aminobenzoic acid, and aspartic acid. Similar results have been obtained with purine-requiring variants of Friedländer's bacillus, and seasonal variation in virulence could be correlated with the nutritional state of the animals.3, 4, 13

Fatigue. It has long been known to the clinician that bodily rest is a valuable adjunct to the treatment of disease, and there is clinical evidence which suggests that resistance to the initial infection may be reduced by excessive fatigue. Experimental evidence on this point is scanty and to some degree conflicting, but it is probable that the unfavorable effect of fatigue on normal physiological well-being is reflected to some extent in an increase in susceptibility to infection. The normal white rat, for example, is highly resistant to anthrax, but when exhausted by work in a treadmill, becomes susceptible. Latent Sal. enteritidis infections in the same experimental animal may be activated by fatigue to such a degree that the outcome is fatal. Exercise has been found to exacerbate intrapleural tuberculous infection in the guinea pig by increasing dissemination of the infection, 12 but, curiously, stressed monkeys have been found to be more resistant to poliomyelitis.71

Other mechanisms operative in the resistance associated with general physiological well-being are obscure. Studies on resistance to the common cold in population groups made by subjecting the incidence data to an analysis of variance among groups have strongly suggested that a constitutional factor, as yet undefined, is operative in the eti-

ology of the clinical infection. There is evidence too that the capacity to maintain effective circulation and the ability to withstand the effects of sudden temperature changes are associated with resistance to experimental infection. The adverse effects of sudden changes in temperature and humidity on the organism, reflected in changes in the nasal mucosa, are, perhaps, a manifestation of temperature shock. Attempts to associate such shock, vitamin deficiencies. fatigue, and other elements of the nonspecific resistance in health with specific defense mechanisms, such as the capacity to form antibodies, have not been uniformly successful.

The external defenses of the organism. The cellular organization of the animal body is a closed system with respect to the outside environment, from which it is separated by the skin, mucous membranes, and intestinal mucosa. These structures, generally impermeable to particulate material of the size of bacteria, constitute the first line of defense against invading microorganisms and one that is, for the most part, highly effective. While mechanical obstruction contributes in no small part to the efficacy of these barriers, both skin and mucous membranes also play an active part in the protection of the organism against bacterial invasion.

The skin. As a rule, the unbroken skin presents a more or less impassable barrier to microorganisms. Bacteria are found normally on the skin between the superficial horny cells, but ordinarily are not able to penetrate deep into the tissues unless favored by some cutaneous injury, such as a wound or burn. Direct penetration of the skin is, however, indicated as the initial route of infection in certain infectious diseases such as Weil's disease (leptospirosis) and possibly syphilis. Also, infection via the ducts of the sweat glands and the hair follicles is strongly suggested in other instances, notably the staphylococcal and streptococcal infections resulting in acne, impetigo, boils, etc., and has, in fact, been demonstrated experimentally with these pyogenic bacteria.

The skin seems not to be a completely inert surface on which bacteria die largely as a consequence of drying. On the contrary, healthy intact skin exerts demonstrable bactericidal activity on those microorganisms not normally present, but the activity is not as pronounced as once supposed. It is re-

garded by some as a consequence of the bactericidal activity of unsaturated fatty acids, especially oleic acid, present in the sebaceous secretions. This activity is inhibited by serum albumin; this may be of some practical significance as regards skin immediately surrounding a burn or other lesion producing a serous exudate. Possibly related is the fungistatic action of the free saturated aliphatic acids in the hair fat of adults. Bacteria normally present upon the skin, such as the white staphylococci, are not appreciably reduced in numbers when swabbed on the clean skin, a fact which probably accounts for their constant presence on the body.

The conjunctiva. Bacteria and dust particles settling in the eves are removed relatively rapidly by the mechanical flushing effect of the tears. The lacrimal secretions contain the enzyme lysozyme, 100 which is also present in certain tissue extracts, is identical with the avidin of egg white, and has been prepared in crystalline form. As described elsewhere (Chap. Three), this enzyme decomposes the cell walls of certain bacteria with the formation of protoplasts, but the protoplasts are ordinarily unstable and the antibacterial effect of this activity is bacteriolytic. Relatively few microorganisms are susceptible to this activity. One, Micrococcus lysodeikticus, is exquisitely sensitive and is destroyed by tears in dilutions as high as 1:40,000.

The nose, nasopharynx, and respiratory tract.^{5, 47, 138} Bacteria and other particulate material present in inspired air are rapidly removed by passage through the tortuous nasal passages lined with mucous membrane to whose moist surface they cling. In this way air is largely freed from bacteria in the upper respiratory passages, those that pass the larynx are caught in the bronchi, and few reach the ultimate ramifications of the bronchioles. The process is so efficient that expired air contains almost no bacteria except those that are expelled in droplets by sneezing, coughing, etc.

The moist film which covers the mucosa of the upper respiratory tract and in which bacteria removed from inspired air and those arriving via the lacrimal secretions are embedded consists of mucus, a thin, highly viscid substance that, in a sense, constitutes a continuous web or membrane overlying the surfaces within the nose, sinuses, pharynx,

and esophagus. This film of mucus is in constant motion as a result of the activity of cilia which sweep the mucus and its bacterial content toward the oropharynx, where it is swallowed. The exchange of mucus is rapid: that covering the posterior two-thirds of the nose is replaced every 10 or 15 minutes, while that over the anterior third is removed every hour or two. Although mucus itself has no bactericidal activity, when combined with ciliary activity it constitutes a remarkably efficient means of ridding the upper respiratory passages of bacteria. 62 Particles above 7 μ in diameter which are inhaled are retained in the upper respiratory passages of the rabbit, about half the particles 3 μ in diameter are similarly removed, and the remainder, plus practically all those 1.5 μ in diameter and smaller, penetrate into the lungs (see also Chap. Ten). The bacteria that penetrate the respiratory passages into the alveoli are phagocytosed by macrophages. 44, 45

Lysozyme is, of course, present in the nasal mucus, and it has been observed that the normal serous secretion of the nose contains a virus-inactivating agent distinct from lysozyme. The activity is virucidal for the influenza and certain other viruses inactivated by sodium deoxycholate, but what part it plays in resistance to infection is not clear.

The mouth, stomach, and intestinal tract. The mouth contains a predominant normal bacterial flora and a minor transient flora. The former consists of microorganisms which have established positions between the teeth, on dental plaques, in the crevices between the teeth and the gums, etc., while the latter represents a constant contamination. Both are subject to constant depletion because of the flushing action of the saliva. Saliva is thought by some to be mildly bactericidal, possibly because of the presence of small amounts of peroxide accumulating as an end product of microbial respiration. The removal of bacteria is, however, largely a mechanical process. The microorganisms flushed to the back of the mouth meet with those from the nose and, with them, are swallowed.

Bacteria reaching the stomach are subject to the strongly acid environment of the normal gastric juice, and there is no doubt that the great majority of them are destroyed there. Some do, however, reach the intestinal tract, perhaps because they are embedded

in solid particles of food and thus protected or because they are able to withstand a short exposure to the bactericidal action of the gastric secretions. Generally, very few viable bacteria are found in the stomach, but the numbers of microorganisms increase in the small intestine with the rise in pH from the duodenum to the ileum. The large intestine contains great numbers of bacteria derived not only from the upper levels of the intestinal tract but also from the multiplication of bacteria present in the intestines as normal inhabitants. As in the respiratory passages, mucus plays an important part in the mechanical removal of bacteria. Here. however, the mucus does not form a uniform coating over the intestinal mucosa but is present as a meshwork. The villi free themselves from particles by movements which bring them in contact with the mucus to which the particles, including bacteria, adhere. The mucus, with the embedded microorganisms, is rolled up into small masses and moved outward by the peristaltic movements of the bowel. Bacteria, then, which enter the mouth and upper respiratory tract are eventually extruded with the feces.

Microorganisms present in the lumen of the bowel, including those making up the normal intestinal flora, are, for all practical purposes, outside the tissues of the body. This flora contributes, in itself, a protective mechanism with which the invading pathogen must compete successfully to establish a focus of infection (see below). Penetration of the tissues from the bowel, as in typhoid fever, is usually via Peyer's patches into the lymphatic system and the blood stream through the thoracic duct. Ordinarily this barrier is a highly effective one, but one of the effects of whole-body ionizing irradiation is a breakdown of this barrier, followed by a generalized invasion of the tissues by bacteria from the intestine, and the immediate cause of death in radiation sickness may be fulminating bacteremia.

The genital tract. The normal genital tract is remarkably free from bacteria. The urethra in both male and female is normally sterile, a consequence, perhaps, of the flushing action of the slightly acid urine. The few bacteria that may be present are confined to the region of the meatus. The normal vaginal secretion is acid and is markedly bactericidal toward most species of bacteria.

Internal defenses. On gaining access to

HORMONES 287

the tissues, the pathogenic microorganism needs to establish and maintain foci of infection, and a number of internal factors in resistance may be distinguished. The relative susceptibility, or resistance of tissues that finds expression as the cytotropism of microorganisms in naturally occurring infections has been described elsewhere (Chap. Four). Other factors include the relevant physiological responses subject to hormonal regulation, and the antimicrobial activities demonstrable in tissues and organs.

Adrenocortical hormones. 58, 114 Hormonal imbalance, and consequent disturbance of a wide variety of functions, is, in general, associated with increased susceptibility to infection. The elements of natural resistance may thus be inferred from effects which result in increased susceptibility. Such effects are not direct, in that the internal secretions affect the microorganism directly, but are concerned, though not necessarily linked, with physiological patterns of response to stimuli such as those provided by the pathogenic microorganisms and their products.

The well-known predisposition of the diabetic to infection, such as gangrenous disease of the extremities, is perhaps to be associated with circulatory disturbance and illustrates the indirection of the relation of hormonal imbalance to natural resistance. Similarly, hypothyroidism results in reduced resistance to experimental tuberculosis, 67 but the effect on the specific immune response is less clear and appears to vary with animal species. 105 Of the hormones, those of the adrenocortical group have been of the greatest interest because of the marked depression in resistance that they produce. In essence, this effect is a consequence of a depressing effect on the inflammatory response and the activity of the cells of the macrophage (reticulo-endothelial) system and emphasizes the significance of these cells in natural resistance. The cellular response to infection is qualitatively the same in the normal and specifically immune animal but is markedly accelerated and accentuated in the latter, and is considered elsewhere (Chap. Fourteen) in this connection. Here the effects of this group of hormones may be regarded as both broadening and modifying the contribution of this system of cells to resistance, as well as indicating the significance of other, probably less important, factors.

The effects of administration of adrenocortical hormones on infections have been summarized by a number of reviewers. The administration of ACTH or cortisone to patients with acute febrile illness usually leads to a prompt defervescence in proportion to the severity of the symptoms, but at the same time there is not only no improvement with respect to the microorganism, and the infection may spread and secondary infections appear almost asymptomatically because of the effect of the hormone. Herpes simplex appears to be one of the exceptions, for topical application of corticosteroids, common in ophthalmology, to herpetic infections of the eye results in an exacerbation of the disease process with the spreading destructive involvement which has often resulted in perforation of the cornea.⁵⁴ Administered to normal animals, these hormones depress natural resistance to experimental infection except in those cases, such as pneumococcal infection in the mouse, where susceptibility is already maximal, and lead to the activation of latent infections if these are present. Further, when given in combination with antibiotics, the efficacy of the chemotherapeutic agents is reduced, emphasizing the significance of the role of host factors in successful chemotherapy. These hormones also accentuate the activity of endotoxins, as indicated earlier in connection with the Shwartzman phenomenon, not by affecting the toxin directly, but by interfering with the cellular response and detoxifying mechanisms. Pre-existing antibody is not affected, nor is the antigen-antibody union, but antibody formation is depressed by these hormones. It is, therefore, not surprising that the appearance of infection is a major reason for discontinuance of cortisone or ACTH therapy initiated for other purposes or that the immediate cause of death in spontaneous hyperadrenalism (Cushing's syndrome) is often infection, and the death rate from this disease has not been altered appreciably since the introduction of antimicrobial chemotherapeutic agents.

Organ and tissue factors.^{52, 112} The possible presence of antimicrobial substances in various tissues and organs is a matter of considerable interest but has not been extensively studied. Two kinds of substances, the one water- or saline-soluble and the other lipid in nature, have been prepared from a wide variety of tissues.

The water-soluble substances found in saline extracts of hog pancreas and thymus and calf thymus are basic polypeptides containing relatively large amounts of lysine or arginine. These substances have, in the aggregate, antibacterial activity on microorganisms such as the anthrax bacillus, streptococci, staphylococci, and tubercle bacilli, differing from one to another in activity. Similar polypeptides are also found in inflammatory exudates in the lung which are active against pneumococci. It is not fully established that all of these substances are not artifacts formed by, for instance, hydrolysis of histones during extraction. Spermine, found in semen and in tissues such as beef kidney, has antibacterial activity against tubercle bacilli. Substances of lipid nature, soluble in organic solvents and slightly soluble in water and saline, have also been isolated from the liver and spleen of rats and guinea pigs and have antipneumococcal activity which is directly related to the susceptibility of the animal species to infection with these bacteria. In most instances such a direct relationship between antimicrobial activity and the susceptibility of the animal from which the tissue extract was derived has not been established.

Interferon, the activity produced by tissue culture cells in response to myxoviruses, and perhaps others, and described earlier (Chap. Four), is not, like the tissue factors described above, pre-existing. While some activity is demonstrable *in vivo*, any contribution such activities may make to natural resistance is as yet uncertain.

Properdin.^{89, 130.} This high-molecular-weight antigenic fraction of human euglobulin, representing no more than 0.03 per cent of the total serum protein, was described by Pillemer and his co-workers as having marked antimicrobial activity against gram-negative enteric bacilli and the Newcastle disease virus when combined in the properdin system consisting of properdin, all four components of complement (Chap. Thirteen), and magnesium ions.

Properdin is inactivated by irreversible combination with a substance known as zymosan. Zymosan is the insoluble residue of yeast cells which have been digested with trypsin and extracted with water and alcohol, and it consists largely of carbohydrate from the yeast cell wall. The properdinzymosan complex selectively inactivates

the third component of complement (C'_3) in the presence of magnesium ions at 37° C. but not at 17° C. The activity is titrated by preparing a serum lacking in properdin but containing C'_3 , by treatment with zymosan at 15 to 18° C. The unknown serum, or body fluid, etc., is then titrated for properdin by mixing varying dilutions with the properdin-free serum plus zymosan, incubating, and titrating for C'_3 in the usual hemolytic system. A unit of properdin is defined as the smallest amount which will remove, as the properdin-zymosan complex, 120 units (50 per cent) of C'_3 .

The nature of properdin is obscure; *i.e.*, whether it is a distinct molecular species having multiple properties or an aggregate of substances is not known. Though originally described as a normal serum component associated with an antimicrobial activity distinct from that of the antibodycomplement system (Chap. Fourteen), it now seems that serum bactericidal activity is dependent upon the presence of antibody occurring as natural antibody in normal serums (Chap. Fifteen). 80 Thus the relation between properdin and natural resistance has become quite uncertain. 102

Cellular response. 29, 46, 96, 137 When the body tissues are penetrated by microorganisms an inflammatory reaction and characteristic cellular response occurs. The area is invaded by phagocytic cells, first heterophils or polymorphonuclear leucocytes, and then by macrophages which can develop into fibroblasts, functional in the repair process. This kind of reaction constitutes one of the most important aspects of resistance, the inflammatory response in the nonimmune animal constituting an element of resistance. In the immune animal this response to the homologous microbial antigen is qualitatively the same, but the reaction is markedly accelerated, and is considered elsewhere (Chap. Fifteen).

Normal flora.⁹⁷ A microorganism somewhat better able to resist the defensive mechanisms of the host but at the same time unable, except when resistance is reduced to a low level, to invade the body tissues, may exist in conjunction with the host as a part of the latter's normal flora. The staphylococci which are able to resist the bactericidal action of the skin are almost invariably present on these surfaces and are regarded as normal inhabitants. The

scanty bacterial flora of the vagina, on the other hand, is composed almost entirely of aciduric bacteria, and the bacterial types present in the intestines are determined to a considerable extent by the type of food material present, *i.e.*, the diet of the host, and by the pH of the various intestinal levels.

Certain kinds of bacteria, such as lactobacilli, spirochetes, various cocci, and the like, exist in the mouth in the interstices between the teeth and in and under tooth plaques and constitute a normal flora characteristic of this region. The bacteria commonly present in the nose and throat consists of still different forms, such as pneumococci. Friedländer's bacillus, and green and hemolytic streptococci. These organisms, while in a sense a normal flora, are not so well established as the flora of some other regions, and the nature of the microorganisms present may be determined in large part by the kind of bacteria which are constantly entering the upper respiratory tract.

As long as the resistance of the host is maintained at a sufficiently high level, the bacteria constituting the normal flora do no harm. If, however, resistance is reduced in some manner, or unbalanced by administration of antibiotics such as the tetracyclines as noted earlier (Chap. Six), the more virulent forms may invade the tissues and set up an infection. The congestion of the nasal mucosa and the consequent interference with ciliary activity and the movement of mucus, which follow the temperature shock of chilling, not infrequently make possible infection by bacteria such as hemolytic streptococci or pneumococci, which are already present.

The normal flora also functions as a part of the resistance complex, especially when the portal of entry of the pathogenic microorganism is the intestinal tract. The pathogen must compete successfully with the normal flora of the bowel to set up a focus of infection in the initiation of the disease process. The protective effect of the normal flora, which may range from competition to antagonism, may be very considerable. The antagonistic relationships involved are complex, being based on the interrelation of the metabolic activities of the competing microorganisms. 33, 49, 83 For example, enteric infection of experimental animals with human enteric pathogens may be difficult to establish, but when the pathogen is made drug-resistant and inoculated intragastrically with the drug, the microorganisms of the normal flora are inhibited, while the drug-resistant pathogen is not and is able to establish an infection.^{32, 76, 77}

Germ-free animals.87, 94, 120 The adaptation of microorganisms to a parasitic existence, indicated by the tendency of long-established diseases to become less severe and at the same time more prevalent, has been considered elsewhere (Chap. Seven). The corollary is whether or not the host, through long association, becomes similarly adapted to the presence of the microorganism, even to such a degree that the relationship becomes a symbiotic one. In certain cases the presence of a parasitic microorganism bears a significant relation to the assimilation of food by the host; such, for example, is the case with respect to the leguminous plants and the root nodule bacteria, and the herbivorous animals and cellulose-decomposing microorganisms of the rumen. Whether the abundant intestinal flora of man and other animals has some analogous relation to the host has been of very considerable interest. 124

Nuttall and Thierfelder early showed that guinea pigs, removed by cesarean section and kept in a sterile environment, survived for 10 days or so, but Schottelius was not successful in rearing bacteriologically sterchicks. Nutritional inadequacies of the chick diet apparently accounted for these results, for it was later shown that chicks could be raised for as long as 40 days in a bacteria-free environment. Modern studies began largely with the work of Reyniers and his colleagues, and have shown that not only chickens, but also a wide variety of animals such as guinea pigs, rabbits, and rats may be reared and bred in the complete absence of bacteria. Nutritional requirements are considerably more complex than for the conventional experimental animal, and natural immunity is limited, illustrating the significant role of the active immune response in the complex commonly considered to be resistance.

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THE EPIDEMIOLOGY OF INFECTIOUS DISEASE

Whatever the pathogenic powers of a microorganism and the efficiency of the defensive mechanisms of the host, an essential preliminary to the production of infectious disease is a meeting of the parasite and its prospective host. In some instances in which the bacterium is naturally saprophytic, it enters the body by accident, so to speak; such, for example, appears to be the case in tetanus, gas gangrene, and similar infections. In most instances, however, the microorganisms that produce disease are more or less closely adapted to a parasitic existence and pass from one animal body to another with only a relatively brief sojourn in the external world. In general, then, the transmission of infection is a process in which the causative microorganism is transferred, either directly or indirectly, from a diseased to a healthy susceptible animal.53

The elucidation of the mechanisms involved in this transfer is a matter of considerable importance. If the sequence of events that precedes infection is known, it may be, and often is, possible to interrupt it at its most vulnerable point and thereby control the spread of disease. Disease is by no means entirely a matter of host resistance and microbic virulence: it is, in a very real sense, the outcome of the interaction of the host and parasite populations. It is at this point that the study of the infectious diseases transcends the microbiology of clinical medicine, with its emphasis on the individual case, and assumes broad biological significance as a problem in interspecies competition.7,37

The equilibrium that tends to become established between the host and parasite populations is an unstable one in that the factors which determine it-i.e., the character of the host population in particular and possibly that of the parasite population. as well as the environmental factors which affect their relationship—are constantly shifting, and the equilibrium ever has a tendency to establish itself at a new level. The shift may be a sudden and violent one whose outward manifestation is an explosive outbreak of disease or, less commonly, may take the form of a gradual increase or decrease in the incidence of the disease.

The factors associated with the maintenance or shift of this equilibrium are the subject matter of epidemiology. 14, 30, 31, 44, 48 The term epidemiology is best regarded in this broad sense and therefore includes the study of the transmission of endemic disease, i.e., disease which has a low incidence but is constantly present in a population, as well as the study of epidemic disease, i.e., disease of high morbidity which is only irregularly present in clinically recognizable form. In this connection it may be noted that the term pandemic is often applied to an epidemic of unusually great proportions. These categories, although useful, are not mutually exclusive; a disease endemic in a community may, at times, attain the proportions of an epidemic and later subside to an endemic level.

Essential to knowledge of the epidemiology of disease are certain characteristics of the etiological agent and of the clinical infection which determine the possible channels of transmission. The more important of these are:

- (1) The route by which the infective agent enters the
- (2) The route by which the infective agent leaves the
- (3) The resistance of the microorganism to the deleterious effects of the outside environment
- (4) Presence or absence of an intermediate host
- (5) The relation between frank, clinically recognizable disease and the discharge of virulent microorganisms from the body

These are most readily and satisfactorily determined by direct study of the etiological agent under controlled conditions as well as the pathogenesis of the naturally occurring disease. When the etiological agent is unknown, however, a first approximation of these properties may be inferred with surprising confidence from the observed epidemiological behavior of the disease. For example, although the famous Broad Street Pump epidemic occurred prior to the discovery of the etiology of cholera, the indirect evidence plainly indicated to Snow that the infective agent left the body in the feces and entered the gastrointestinal tract via the contaminated well water.40

The carrier. With regard to the last characteristic listed above, additional explanation is desirable. In the early days of microbiology it was assumed that the contact of host and parasite could have only one or the other of two outcomes; either no infection occurred, owing presumably to high resistance on the part of the host, or clinically characteristic disease developed in the individual. Subsequently it became clear that an intermediate state, the establishing of a symptomless infection, may occur. Such infections are, of course, inapparent and concealed and may be demonstrated only by isolation and identification of the infectious agent. An individual so infected is termed a carrier.

Two types of carriers are commonly differentiated, the casual carrier who harbors the microorganism temporarily, a matter of a few days or weeks, and the chronic carrier who remains infected for a relatively long time, sometimes throughout life. Such individuals serve to disseminate the infectious agent. In the first group are the great majority of carriers of the diphtheria bacillus, meningococcus, pneumococcus, certain streptococci, etc., and the second includes carriers of certain of the enteric bacilli, especially the typhoid bacillus. A third type of carrier is often differentiated, the convalescent carrier, who remains infected for a greater or lesser length of time after recovery from the disease. These last do not fall into the category of concealed infections. Sharp separation is sometimes not possible, however, for the casual or chronic carrier may. in fact, be convalescent from the disease in a form either atypical or so mild as to go unrecognized (ambulatory cases).

While it is now commonplace to recognize the existence of the carrier state, the implications of the general principle that infection may occur without disease are frequently neglected. Thus it follows that clinically apparent infections may constitute only a part, in some instances only a very small part, of the infections continually taking place. Clearly, then, if an important proportion of the infections are of the concealed type, a reasonably accurate estimate of the extent to which the infection is disseminated in the host population cannot be arrived at on the basis of cases of the disease. The implications of this are several. For example, diseases which occur sporadically and do not seem to be easily or often transmitted from the sick to the well, such as poliomyelitis, meningococcus meningitis, and pneumococcus pneumonia, may be as widely disseminated and readily communicable as measles or the common cold, but the clinically distinctive disease is the exception rather than the rule. Furthermore, the rise or decline of infectious disease or its age or geographical distribution may not reflect a corresponding variability in the prevalence of the infection but rather may be a consequence of variation in the casecarrier ratios. Though here inferred from the observed occurrence of the carrier state, none of these possibilities remains purely hypothetical for all have been found to exist. Thus, carriers of virulent pneumococci do not occur predominantly in the higher age groups, or diphtheria bacillus carriers in the schoolchild, where the morbidity of these diseases is highest; diphtheria bacillus carriers are as common in the tropics as in temperate climates despite the relative rarity of clinical diphtheria in the hot climates; and so on. In many other cases, such as that of poliomyelitis, such a situation is suspected but technically difficult to prove. It will be obvious, therefore, that the recognition of the carrier state and its implications is basic to sound epidemiological thinking and of primary importance to the understanding of the mechanism of spread of the infectious diseases.

EPIDEMIOLOGICAL TYPES OF INFECTIOUS DISEASE

On the basis of such fundamental information, the infectious diseases may be separated into a number of epidemiological types which, despite certain limitations, serve to illustrate the diversity of ways in which infection may be disseminated.⁵ A rough classification, based on the assumption that the human being is the recipient of infection and that the control of diseases of man is the point at issue, follows:

- (1) Diseases of lower animals transmissible directly to man (rabies, tularemia, glanders, etc.)
- (2) Diseases of animals or man transmitted by insect vectors in which
 - (a) The insect acts as a mechanical carrier e.g., the housefly (typhoid fever)
 - (b) The parasite multiplies in the insect vector (bubonic plague)
 - (c) The parasite is transmitted from one insect generation to the next by egg infection (spotted fever)
 - (d) The parasite undergoes a portion of its life cycle in the insect (malaria)
- (3) Diseases of animals or man transmitted indirectly (a) By water (the enteric infections such as typhoid fever and cholera)
 - (b) By milk (scarlet fever, bovine tuberculosis, undulant fever, etc.)
 - (c) By food (typhoid and paratyphoid fevers)(d) By inanimate objects or fomites, such as books and towels (scarlet fever, diphtheria, and the like)
- (4) Diseases of man transmitted directly
 - (a) By infective droplets—airborne infection (the respiratory diseases and others)
 - (b) By direct contact (the respiratory and veneral diseases in particular)

The epidemiology characteristic of a given disease shows, on the one hand, certain and sometimes close relationships to other similar infections, and, on the other, a certain variability which arises as a result of transmission in more than one way. The enteric infections, cholera, typhoid fever, and the bacillary dysenteries, are similar to one another in epidemiology but are

quite different in this respect from the respiratory diseases or the insectborne diseases. The epidemiology of typhoid fever, however, is variable within limits and depends to some degree upon whether the disease is waterborne, milkborne, or transmitted by food or contact. In the case of waterborne typhoid, the drinking water supplies the connecting link, and the ensuing epidemic, usually explosive and limited to the area supplied by the contaminated water, shows no respect for age, sex, or economic status. Milkborne typhoid, on the other hand, is geographically limited by the route by which the infected milk is delivered, exhibits an increased incidence in the lower age groups and among females and is somewhat more frequent among those of higher economic status. Foodborne infection is still more limited, often within a family group, with no discrimination as to age or sex.

Variation in the epidemiological character of a disease may also occur with changes in the behavior of the host population. For example, the age incidence of poliomyelitis has altered in the past few decades, with a shift to the higher age groups and disappearance of the marked preponderance in young children, coupled with an increasing frequency of epidemic outbreaks of the disease. The shift has been greater in the North than in the South in this country, greater in rural than in urban areas, and greater in the United States than in other countries. This shift is commonly interpreted as evidence of postponement of effective contact with the virus, and there is some evidence that this is associated with economic status; i.e., the shift is more marked in the higher economic brackets.3 The shifting pattern in the behavior of poliomyelitis is more complex than this, and is discussed in more detail elsewhere (Chap. Thirty-nine).

Airborne infection. Many of the diseases of man are transmitted as airborne infection in which suspended infectious material is inhaled. In contrast to the indirect transmission of disease by, for example, agencies such as water and milk, this kind of link between the source of infection and its recipient is not readily broken, and it has usually not been possible to control airborne infection in this way to any practical extent. Under natural conditions, the in-

fectious material may occur in finely dispersed form in air originating directly from the source of infection, or it may be present in dust that, on resuspension in air, is inhaled.¹⁸

Infectious clouds. It was postulated many years ago by Pflugge that diseases of the upper respiratory tract could be transmitted by droplets containing the microorganisms and expelled from the mouth and nose during coughing and sneezing. The droplets he studied were relatively large, greater than 0.1 mm. in diameter, and seeded the air for only short distances before falling to the ground. Since epidemiological considerations demanded airborne infection effective at considerable distances, Pflugge's "droplet infection" appeared relatively unimportant. In the early 1930's, however, the matter was reinvestigated, especially by Wells and his associates, and it was found that Pflugge's evidence was incomplete, in that under usual conditions of humidity droplets smaller than 0.1 mm. in diameter are rapidly reduced in size by evaporation to leave suspended nuclei containing microorganisms that may remain viable and infective for a matter of hours. The process of expulsion of such droplets in coughing, sneezing, and talking to give rise to such suspensions has been studied by Jennison²⁰ and is illustrated in the accompanying figure. An example of the significance of such spread is the description of "cloud babies," infants who disseminate infectious clouds of staphylococci, and play a significant part in the dissemination of infections in nurseries.11

Interest in inhalation infection markedly stimulated during World War II because of the possibility that airborne infectious materials might be used as biological warfare agents,22 and the subject has been studied in considerable detail.³⁶ Infectious agents may be finely dispersed in air under controlled conditions in the laboratory by means of atomizers or nebulizers, and the cloud so produced may be studied with respect to viable microorganisms, infectivity, and the like.55 It has been found that survival of bacteria in the ambient cloud is conditioned by relative humidity and the nature of the original suspending medium. While many of the microorganisms may be killed, it is possible to generate clouds containing large numbers of viable

cells. The infectivity of such clouds of pathogenic microorganisms may be tested by exposing experimental animals in appropriate chambers, 9, 19 and it has been found that infection is readily produced by inhalation of clouds of appropriate pathogens.

Assuming the presence of viable microorganisms in sufficient number in the cloud. their distribution following inhalation is determined primarily by particle size. As pointed out elsewhere (Chap. Nine), the upper respiratory tract is a highly efficient mechanism for the removal of particulate matter suspended in inhaled air through impingement of such particles on the wet mucous surfaces. In general, large particles are removed in the nasal area, while very small particles may not be retained at all, but exhaled. Eighty per cent of particles 1.8 μ in diameter, 50 per cent of 4 μ particles, and 20 per cent of 12 μ particles penetrate the nose; when the nose is bypassed by mouth breathing, 23 per cent of 1 μ particles, 38 per cent of 2 μ particles, 52 per cent of 3 μ particles, and 68 per cent of 5 μ particles are retained in the lungs.³² Total body retention is, however, greater than indicated by examination of the respiratory tract alone. Inhaled bacteria are also present in the esophagus and stomach. By the use of radioactive phosphorus as a tracer, it has been found¹⁵ that there is about a 30 per cent retention of 1 μ particles in the respiratory tree but a practically complete whole body retention of the theoretically inhaled dose, with the remaining 70 per cent predominantly in the gastrointestinal tract. The occurrence of inhaled material in the gastrointestinal tract is, of course, a consequence of the removal mechanism described elsewhere (Chap. Nine) in which mucus secreted in the upper respiratory tract is swallowed, and contained bacteria are eventually excreted with the feces.

A wide variety of laboratory procedures—ranging from pipetting and decanting of supernatants after centrifugation to opening freeze-dried cultures and the operation of blenders—generate aerosols. Infectious aerosols so produced probably account for the majority of accidental laboratory infections, and their control is a matter of both proper equipment and technique.^{34, 42, 52}

Infectious dust.³⁸ The source of pathogenic bacteria in dust is two-fold: the settling out of droplets containing them that are too

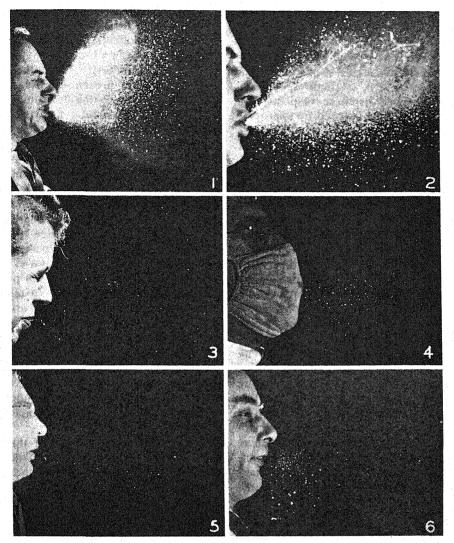


Figure 59. The atomization of mouth and nose secretions demonstrated by high speed photography. 1, a violent sneeze in a normal subject; note the close approximation of the teeth, resulting in effective atomization. 2, head cold sneeze; note the strings of mucus and the less effective atomization of the viscous secretions. 3, a stifled sneeze. 4, sneeze through a dense face mask. 5, cough; note the smaller discharge than in the uninhibited sneeze. 6, enunciation of the letter "f." (Jennison.)

large to remain suspended in air any great length of time; and contamination of objects, especially textile products such as handkerchiefs and blankets, with infectious secretions that, after drying, become infectious dust. For example, contamination of the environment with Streptococcus pyogenes by infected persons has been shown to be in part a contamination of dust. Present evidence suggests that the inhalation of airborne bacteria in dust may be quantitatively more important than that of directly expelled particles; i.e., dust is probably a more

prolific and constant source of contamination of air. Control measures directed toward the suppression of dust, such as the oiling of blankets and floors in infectious wards in hospitals, have been more effective in the control of infection than attempts to destroy bacteria in air, as by ultraviolet irradiation.

THE MICROBIAL POPULATION

The well-known variation in the severity and "contagiousness" of the infectious

diseases is a consequence of corresponding variation from one species of pathogenic bacteria to another in their ability to invade the body tissues and, once established, to produce clinical disease. As indicated elsewhere (Chap. Seven), a single species is potentially variable in these respects, for such variations can be induced by appropriate experimental manipulation. The possibility of such intraspecies variation in a bacterial population existing under natural conditions is one that has intrigued students of infectious disease for many years.

It is tempting to account for the genesis and rise of epidemic disease by assuming that the causative agent of a disease of endemic proportions gains in virulence by successive passage from person to person until its pathogenicity is so enhanced that an epidemic ensues. Similarly, a sojourn in a host population containing an increasingly large proportion of immunes might be expected to result in a diminution in the virulence of the microorganism and consequent subsidence of the epidemic. Furthermore, successive epidemic waves might conceivably result from periodic fluctuations in the virulence of the parasite. Unfortunately for such an explanation, there is little or no direct evidence that alterations in virulence play an important part in the evolution of single or secondary epidemic waves, and, in nature, microbial virulence appears to be a relatively stable character.

On the other hand, differences in the severity of a single disease from one epidemic to another are, in part, attributable to the existence of strains of the infectious agent which differ from one another in virulence. Benign and malignant smallpox referred to above is a case in point, and some workers believe that some strains of the diphtheria bacillus produce a more severe disease than others (Chap. Thirty). Although the bacillus varies from one strain to another, available evidence indicates that within a single strain virulence does not fluctuate to a demonstrable degree.

Possibly attributable in part to alterations in bacterial virulence are the changes in morbidity and mortality of some diseases such as scarlet fever, syphilis, and tuberculosis over long periods. In the case of scarlet fever the 25 years prior to 1830 was a period of very low death rates and was followed by a 40-year period of high death rates. Since

then the death rate has declined, and, though the incidence remains high, the case fatality is relatively low. In other diseases only a decline has been observed. Syphilis is no longer the scourge it was in the sixteenth century, and the present decline in tuberculosis began before the institution of preventive and therapeutic measures. In still other diseases, such as measles, no such long-term alterations in prevalence have been observed. Information is as yet too limited to assess these phenomena; possibly in some diseases there are long-term periodic fluctuations in bacterial virulence (this may be an artifact and represent only variations in the prevalence of virulent "epidemic" strains of the parasite), while in others an adaptive reduction in virulence or increase in resistance on the part of the host or a combination of both may play a part. Perhaps the most elegant example of this phenomenon has been illustrated by the attempt to control the rabbit population in Australia by the introduction of myxomatosis virus. Initially the rabbit population declined sharply, but with adaptation of the host and parasite, an equilibrium became established with essentially complete defeat of the initial objective. 13

In general, it may be said that, in the short view, the bacterial population, as it exists in nature, is remarkably stable insofar as its ability to produce disease in a host population is concerned. Although the severity of a disease may, and often does, vary from one epidemic to another, variation in virulence is not an important factor in the single epidemic wave. Over long periods, however, alterations in virulence may contribute to the changes in morbidity and mortality observed in some of the infectious diseases.

THE HOST POPULATION

In contrast to the relative stability of the bacterial population, the human population is highly variable in its resistance to infection, and the variation, attributable to both intrinsic and extrinsic factors, is not infrequently of such magnitude that its consequences are of considerable practical importance.

Since a population, human or infrahuman, is composed of individual organisms, it

follows that its character is determined by the nature of these individuals and their relations to one another, and its reaction to an external influence is expressed in terms of the aggregate of the reactions of its members. The response of a human population to an infectious disease is, of necessity, measured in terms that are composites of the responses of the individual members of the population - in short, by some method of counting. Such counting is, of course, the basis of statistics, and the statistical method, with its ramifications and refinements, is a powerful tool which makes possible the study of the response of human populations to disease – a response which is measured in terms of rates, ratios, life tables, and similar numerical devices.10

Intrinsic factors. Of the intrinsic factors which determine the response of a human population to an infectious disease, one of the most important is age distribution. The quantitative predominance of the lower age groups characterizing an immature population declines with population growth while the higher age groups correspondingly increase. As a consequence, the diseases of childhood and early adult life, such as

diphtheria, tuberculosis, and the like, are relatively prevalent in an immature population but become progressively less so with the passage of time, while the diseases of old age increase in incidence with the maturing of the population. The frequency of an infection is expressed as a rate, either as the number of persons infected in a given unit of time, i.e., incidence, or as the total number of persons infected at any one time, i.e., prevalence. Correction of morbidity and mortality rates is a practical necessity and is made either by the use of specific rates, i.e., the proportion of cases or deaths within a specified age group, or by the use of standardized rates which are not the observed rates but rather what the observed rates would be if the age distribution of the population were that of a standard or reference population.*

^{*}Two standards have been used in Europe: one, the population of Sweden as it existed in 1890; and the other, the population of England and Wales as shown by the 1901 census. The first is standard in age distribution only, while the second is standard for both age and sex. In this country the population of the entire country is usually taken as a standard, and the populations of states or other portions of the country compared or adjusted to it.

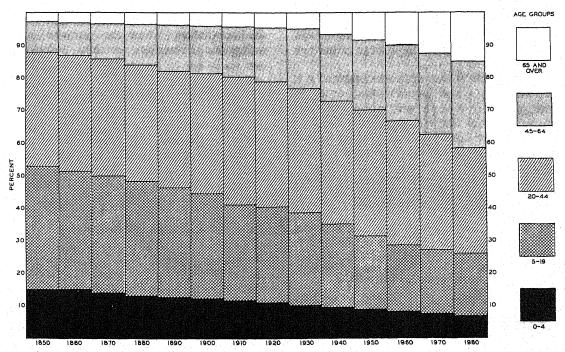


Figure 60. The age distribution of the population of the United States, enumerated from 1850 to 1960 and projected to 1980. (Data from the U. S. Bureau of the Census: Decennial Censuses, and Current Population Reports, Series P-25, No. 241, 1962. Compiled by the Population Research and Training Center of the University of Chicago.)

The sex distribution of a population and its racial composition are of somewhat lesser practical significance, although in some communities in the United States the high mortality rate of the Negro has necessitated the use of race-specific mortality rates.

Extrinsic factors. The extrinsic factors which alter the resistance of a host population to the spread of disease may exert their effects in either or both of two ways: first, by influencing the resistance of part or all of the individuals comprising the population, and, second, by influencing the relationships between individuals. Perhaps the most important factor in the first category is active individual immunity. If a sufficient portion of a population is immune to a disease as a result of artificial inoculation or recovery from an attack, the resistance of the entire group to epidemics of that disease is of a high order, a phenomenon which has been termed herd immunity. Other factors may reduce the resistance of the individual; in times of stress or calamity, for example, when relatively large groups are undernourished, fatigued and exposed to inclement weather, epidemic disease may spread with great rapidity.

Equally important to the resistance of a population to epidemic disease are the factors which determine the interrelationships of its members. Crowding in large gatherings

or the enforced close association arising from inadequate housing facilities obviously provides opportunity for the dissemination of respiratory and other diseases transmitted directly from man to man and, as well, certain indirectly transmitted infections, such as louseborne typhus fever. Similarly, the spread of enteric infections is, to a large extent, dependent upon sanitary facilities and the solution of the twin problems of water supply and sewage disposal. Group practices which support a large rat population make bubonic plague a potential menace, and the presence of large numbers of mosquitoes of the appropriate species allows the wide dissemination of malaria and yellow fever. These and other factors, political, sociological, or economic in nature, obviously exert no small influence on the resistance of a population to the spread of disease.39

THE INTERACTION OF HOST AND PARASITE POPULATIONS

It will be clear from the foregoing discussion that the interaction of the host and parasite populations is a highly complex phenomenon. Even assuming that the parasite population, when the parasite is a pathogenic bacterium, remains relatively

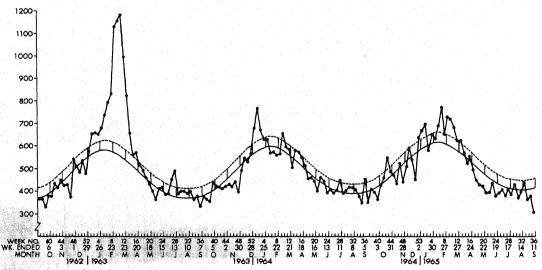


Figure 61. The effect of season on infectious disease as illustrated by the seasonal variation in death rate from pneumonia-influenza in large cities in the United States over a three-year period. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare.)

constant in its ability to produce disease, the resistance of the host population is in a constant state of flux, and the equilibrium between the two is rarely a "steady state." As has been indicated, the relation between host and parasite populations is a part of the general problem of interspecies competition and has been studied at length. The infectious diseases of man constitute a series of special cases of the host-parasite relationship, differing from one another with respect to mode of transmission, incubation period, period of infectivity, immunity, case fatality, etc. The studies on infectious disease have taken two forms: one, the theoretical analysis of epidemic spread; and the other, the experimental investigation of controlled epidemics among populations of laboratory animals, i.e., experimental epidemiology.

Theoretical analysis. 29, 33, 41, 47 Theoretical treatment of the incidence of infectious disease in time consists of the formulation of mathematical models based upon the probability of contracting disease, independent of hypothetical variations in the virulence of the microorganism or the resistance of the host population other than as acquired immunity. A number of variables must be taken into account, including the effective contact or dose required to produce infection, the homogeneity of mixing infected and susceptible persons, infectivity of the recipient with respect to the latent or incubation period and to the onset and defervescence of the disease, and the effectiveness of the immunity associated with recovery. Of these, it is generally assumed that recovery from the disease gives an effective immunity, but the way in which the other relevant factors are presumptively defined prior to formulation in mathematical terms leads to two kinds of approaches, the one deterministic and the other stochastic.

The deterministic approach is based on the assumption that when the numbers of susceptible and infected persons are known, together with rates such as those of attack, birth, and death, any future state of the host population can be precisely determined. From the stochastic point of view, the variables are regarded as probabilities; Bartlett's formulation, for example, is based on the assumption that infection and removal of susceptibles are random events in time, and he developed the probability generating

functions for these two variables—the numbers of susceptible and infected persons.

From another point of view, mathematical models are of two kinds, the point-infection type and the continuous infection type. The earlier models, including the Ross malaria equations, and the binomial expansions of Reed and Frost, of Soper, and of Greenwood, are of the point-infection type in that the period of infectiousness is contracted to a point, and the development of successive stages, or generations, of cases of disease following the introduction of infection into a susceptible population is described by the successive terms of a binomial expansion.1 More than one probability value may be functional in a given epidemic by inhomogeneity of mixing, e.g., that within families of various sizes and within the population as a whole, leading to binomial chains, and a stochastic element is introduced when the frequency distribution of these probabilities is taken into consideration.

The constant infection model of Kermack and McKendrick^{21, 28} may be illustrated in deterministic form, *i.e.*, by using constant infection and removal rates. In a homogeneously mixing group of n individuals, at time t there are x susceptibles, y infectious individuals, and z who are isolated, dead, or recovered and immune; then:

$$x + y + z = n$$

If a constant infection rate is designated β , and a constant removal rate γ , the number of infections in time dt is $\beta xydt$, and the number of removals is γydt , and the course of the epidemic is represented by the differential equations

$$dx/dt = -\beta xy$$

$$dy/dt = \beta xy - \gamma y$$

$$dz/dt = \gamma y$$

Approximate solution gives the rate of change of the total number of cases, or the epidemic curve, as

$$dz/dt = (\gamma^3 u^2/2\beta^2 x) \operatorname{sech}^2 (1/2u\gamma t - \theta)$$

and the total size of a small epidemic is approximately

$$(2\gamma/\beta x_0)(x_0-\gamma/\beta).$$

When t = 0 and the entire population consists of susceptibles, *i.e.*, x = n, unless $n > \gamma/\beta$, no epidemic can start, and the

relative removal rate, $\rho = \gamma/\beta$, gives the threshold density of susceptibles. When n is greater than the threshold density, e.g., $n = \rho + \nu$, the total size of an epidemic will be 2ν , reducing the initial density of susceptibles, $\rho + \nu$, to $\rho - \nu$, a value as far below the threshold ρ as it was above it initially. This is the Kermack and McKendrick Threshold Theorem and is illustrated in the accompanying figure.

When the continuous infection type of model is treated stochastically it becomes much more complex mathematically and will not be described here, but there is reason to believe that the stochastic model is a powerful tool in the study of the dissemination of infection. The development of such models serves to introduce an element of precision into epidemiological thinking, and their application has indicated their general validity. 4, 8, 17, 50 In any case, it is clear that the spread of disease and the generation of epidemics may be accounted for by application of the principle of mass action without recourse to unfounded assumptions of systematic variation in the virulence of the microorganism.

Herd immunity. The nature of herd immunity will become clear at this point, for the higher the proportion of immunes

in a population the smaller the probability of effective contact between case and susceptible; i.e., many of the contacts will be with immunes, and the population exhibits a group resistance to epidemic disease which may be of such a high order that an epidemic is no longer possible and the disease smolders in an endemic form as a result of the importation of new cases or the persistence of infection in healthy carriers whose contacts will give rise to an occasional case. A susceptible member of such an immune population, then, enjoys an immunity that is not of his own making but arises as a result of his membership in the group.

During the course of an epidemic, the decline in number of susceptibles to the threshold density coincides with the peak of the epidemic wave, and the incidence of new cases declines because the disease cannot reproduce itself. The total number of cases in the epidemic will, as described above, be twice the number of susceptibles in excess of this threshold density present at the beginning, and following subsidence of the wave the population is left with a greater or lesser degree of herd immunity.

Such precise relationships are only approximated in nature. In practice the thres-

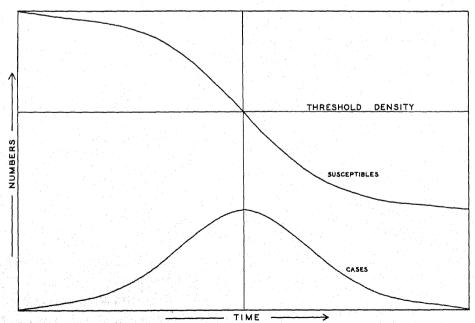


Figure 62. Diagrammatic representation of the course of an epidemic wave in terms of number of cases (lower curve) and number of susceptibles (upper curve). Note the coincidence of the peak of the epidemic wave with the threshold density of susceptibles. (After McKendrick.)

hold density fluctuates and is a function in part of dosage. For example, immunization of 30 per cent of preschool children suffices to control epidemic diphtheria, but the proportion is much higher in schoolchildren and 70 per cent is the commonly accepted level of herd immunity required to control epidemic diphtheria. The higher proportion required in older children is perhaps a consequence in large part of increased exposure to infection. Similarly, Sabin has estimated that immunization of 70 per cent of children should suffice to eliminate epidemic poliomyelitis.

When the prevalence of a disease is high. a given individual will be subjected to a greater number of bacteria per unit time; some, previously immune to smaller doses. will become susceptibles under such circumstances and the "effective concentration," so to speak, of immunes declines with consequent effect on the herd immunity to epidemic disease. This effect of dosage on the host population has been termed infection pressure and is a matter of considerable practical importance. This, in effect, alters the infection rate β , taken above as invariant, since it is related to dose per unit time, and alters ρ , the threshold density. Successive epidemic waves may, then, occur as a consequence of increased infection pressure as well as through the accumulation of new susceptibles (see below). Recurrent waves of a disease that is a clinical but not an etiological entity are, of course, another matter.

Long-term relationship. The question of the relationship between an infectious disease and a susceptible population over a long time is somewhat more complex. Mathematical treatment such as the Ross malaria equations and Martini's equations for immunizing diseases26 is too far removed from reality to have much practical significance, principally because the mathematical approach requires that the parameters remain constant over long periods. This condition is probably rarely if ever satisfied; it is well known, for example, that within the last century both diphtheria and scarlet fever, previously of low prevalence, took on a malignant character and higher prevalence for some decades and then within the last 50 years have gradually declined in both prevalence and severity. It is of some interest, however, that the differential equations of Martini, for example, show one stable equilibrium at the origin and another at a positive value which is approached by a series of oscillations above and below the final state of equilibrium so that a series of epidemic waves appears. These solutions suggest that (a) under certain circumstances a disease may tend to die out and (b) under other circumstances a disease will reach an equilibrium after a series of epidemics of decreasing severity. Such periodicity in the incidence of many infectious diseases is well known; one of the best examples is that of measles, which recurs in epidemic form at approximately two-year intervals. So far as is known, however, no infectious disease occurring under natural conditions shows a damped periapproaching invariancy though odicity it has been found to occur in experimental epidemics in which the immigration rate of the new susceptibles is very high.

A disease will, of course, die out if it does not reproduce itself, i.e., if the bacterium is present only in the active case and each case does not, on the average, give rise to a second case. It is not unlikely that some diseases are dying out in this way. It has been suggested, for example, that the decline in tuberculosis is due in part to inability of the disease to reproduce itself, a process accelerated by isolation of active cases. The second suggestion is of interest in connection with the epidemic periodicity of certain diseases such as measles. It is obvious, of course, that following an epidemic a new crop of susceptibles appears, and when their numbers reach a sufficiently high level, a new epidemic ensues, and so on ad infinitum. It is unlikely, however, that these epidemic waves are damped as predicted by theoretical equations, for subtle variation in the complex of factors that are oversimplified as parameters will wipe out the damping effect. It will probably be many years before predictions regarding the future of infectious diseases attain a status better than that of guesses.

EXPERIMENTAL EPIDEMIOLOGY^{16, 49, 51}

The information that may be derived from observation of naturally occurring disease, i.e., descriptive epidemiology, is limited since the observer has no control over the process, and for all practical purposes the experiment is carried out for, instead of by, him. In experimental epidemics, however, the conditions may be adjusted as desired, and it may be anticipated that such experiments will be highly informative.

The study of epidemics of infectious disease developed in populations of laboratory animals under controlled conditions has been carried out by Topley, Greenwood, Wilson, and others in England and by Webster and his colleagues in the United States. These experimental epidemiological studies have been confined, for the most part, to the study of the dissemination of Salmonella typhimurium (aertrycke), Sal. enteritidis, Pasteurella muriseptica, and the virus of ectromelia (mousepox) among populations of mice. Mouse typhoid and pasteurellosis are regarded as analogous to human diseases such as typhoid fever in which there is an imperfect immunity and carrier state, and ectromelia as analogous to human diseases in which a solid immunity is developed, such as diphtheria. The extent to which the analogies may be carried, and to which the conclusions reached are applicable to the considerably more complex human population as it exists under natural conditions, is open to some question; nevertheless, these studies, as yet in their infancy, have yielded valuable information.

The experiments which have been carried out were of two general types. The closed epidemic was produced in a population of mice, often about 50 in number, by the introduction of infected animals. The other type of experiment was carried out in an infected mouse population recruited by continuous immigration, *i.e.*, mice were added at regular intervals, the rate varying from one mouse every three days to six mice per day. The results may be summarized briefly:

(1) The epidemic wave initiated in the closed population by the introduction of infected individuals closely resembled those observed in the human population.

(2) The effects of dispersal of the infected population of the closed epidemic into large or small groups at various times during the development of the epidemic were studied. The time at which the population was dispersed was found to be of primary impor-

tance; the later the dispersal was effected, the less favorable the result, and after the peak of the epidemic wave was reached it continued unchecked even though the population was dispersed to individual mice. This is of particular interest since fleeing from an epidemic has been a popular method of escaping infection (cf. The Decameron). In this connection the results of dispersal of children from the industrial centers of England in 1939-1940 may be noted. A sharp reduction of 40 per cent or more in diphtheria morbidity occurred in the evacuated towns, together with an increase of 60 to 70 per cent among local children in reception centers, the latter returning to normal within six months. Similarly, the biennial periodicity of measles was broken by this dispersal.

(3) The effect of active immunization on the epidemic was not so great as might be supposed. If the mice were immunized prior to the epidemic, a favorable effect was apparent and more marked in ectromelia than in Sal. typhimurium infections. Immunization undertaken after the initiation of the epidemic was without effect.

(4) In the mouse population recruited by continuous immigration a series of epidemic waves developed, the frequency of which was directly related to the rate of immigration. Thus, with a low rate, such as one mouse every three days, epidemic waves were separated by periods of remission in which no deaths occurred. As the immigration rate was increased, the waves occurred with greater frequency, and with the addition of six mice per day there were no periods of complete remission, the epidemic waves taking the form of periods of increased mortality.

(5) With high immigration rates, the peaks and troughs in the daily death rate became less and less pronounced after a year or more, both the rate and total population tending to become invariant. Thus it was possible to approximate experimentally the damped periodicity and eventual equilibrium predicted on theoretical mathematical grounds (see above), and the experimental host population could be brought into approximate equilibrium with the parasite population.

(6) An inadvertent experiment with a population recruited by a high rate of im-

migration proved to be of considerable interest. The mouse population had reached the equilibrium indicated above by summer. when there was a heat wave of unusual intensity in London. There were many nonspecific deaths (mice are highly susceptible to heat), followed by violently fluctuating specific mortality. In the course of time an equilibrium was again reached but at a considerably higher death rate and decreased numbers than formerly. The parasite was shown to be unaltered in virulence, and the immigrants had not, of course, had the heat experience. Hence the host-parasite relationship per se was altered by an environmental "catastrophe." The relationship of these observations to the long-term relation between the human population and infectious disease is of interest. If it be assumed that the day of the mouse is equivalent to 30 days in the life of man, over the four years taken by this experiment the following were observed: (a) a period of stability with low regular death rates and growing population (ca. 600 days) equivalent to a human experience of slightly less than 50 years—this is longer than the 25-year period ending about 1830 during which the death rate from scarlet fever was relatively low: (b) a period equivalent to 15 years of severe and repeated epidemic waves; and (c) a period equivalent to some 30 years of relatively high mortality in a more or less stable state of equilibrium in a population reduced in numbers—the post-1830 epoch of virulent scarlet fever lasted more than 40 years.

(7) The mortality rates of the mice were intimately related to the length of time that they were members of the infected population; the rate rose rapidly to a peak in the early days of cage age and, although it declined slowly with the passage of time, to a greater extent in ectromelia than in mouse typhoid or pasteurellosis, the survivors of one epidemic wave may be the victims of another. The removal at regular intervals of a number of mice equal to the number added at the same intervals markedly altered the trend of these mortality rates: under these circumstances, the initial peak of mortality was lower, but the rapid decline was not apparent and the mortality rate remained at a high level. Removal was of considerable advantage to the individual mouse; in general, the earlier the removal the greater the advantage, except during the decline of an epidemic wave, when isolation did not increase the chances for survival of individual animals.

Although the evolution of the single epidemic wave in the experimental studies closely resembles its counterpart in the human population, the inability to control experimental epidemics by immunization is at variance with observations on epidemic disease in human populations. Some human diseases, such as diphtheria and smallpox, can be restrained from assuming epidemic proportions by active immunization of a sufficiently large number of the individuals making up the population. Whether or not this and certain other minor discrepancies prove to be real or illusory with future work. a number of pertinent suggestions have come out of these investigations. The response of the experimental host population in terms of mortality rates and cage life expectation cannot be interpreted as vet in some cases: in others the interpretation is questionable.

Some of the experiments, however, have yielded clear-cut results that bear directly upon the interaction of host and parasite populations in nature. Perhaps the most important of these is the experimental demonstration of recurring epidemic waves resulting from the continuous addition of new susceptibles to the infected population. In spite of the fact that such a sequence of events is predicted by theoretical epidemiology, as indicated above, the repeated flaring up of a disease of man or domestic animals, thought to be stamped out in the interepidemic periods, is most often taken as evidence of the re-importation of infection. The experimental demonstration of this phenomenon and the indicated futility of quarantine in the control of a disease which has become widely disseminated (only when a disease is not present, or present in limited effective foci, can quarantine and isolation procedures be effective) are, clearly, of considerable significance.

EPIDEMIOLOGICAL DATA AND THEIR INTERPRETATION²⁷

The purposes of epidemiological study are three-fold: first, to indicate the nature of the infective agent, its source, and modes of transmission when these are not fully established otherwise, *i.e.*, on an experimental basis; second, to extend this information into a corresponding general theory of the epidemiology of the disease; and third, to determine in detail the local conditions which favor or control the dissemination of the infection in a given area or community. For these purposes four general types of information, usually simple in character but wide in extent, are required.

Geographical distribution. The area in which the disease occurs and the regularity of its geographical distribution are highly informative. Thus, the general occurrence of a disease indicates that environmental conditions, including fauna, climate, etc., peculiar only to parts of the area are not essential to its transmission. Similarly, restriction of the disease within geographical limits indicates that special environmental conditions are necessary for its dissemination; these may include crowding, presence of an insect vector, water supplies, proximity to reservoirs of infection, etc. In general, a uniform distribution of the disease is indicative of a simple method of transmission, as in the case of measles, whereas an irregular distribution, such as that of spotted fever, implies a more complex process dependent upon a source of infection or conditions necessary to transmission that are correspondingly irregularly distributed.

Prevalence of disease. The prevalence of the disease suggests the source of infection. A high rate, such as that of measles, indicates that the observed cases are the most important, if not the only, source of infection. Conversely, sporadic distribution of widely separated cases implies the existence of a concealed reservoir of infection such as the casual or chronic carrier or some of the lower animals.

Seasonal distribution. The seasonal distribution is also informative when considered together with other epidemiological features of the disease. Thus, a seasonal occurrence may be consistent with hypothesized insect transmission, or it may eliminate an insect vector as the sole transmitting agent.

Age distribution. The age distribution of the disease is suggestive as a corollary to other epidemiological characteristics, and often aids in their interpretation. Thus, a disease occurring for the most part in the

early years of life, such as measles or diphtheria, often shows differences in age incidence between urban and rural areas which are explained by the more frequent occurrence of immunizing infections, apparent or subclinical, in the more crowded areas. Since similar differentials are apparent in poliomyelitis, the age distribution of this disease, together with other of its epidemiological features, supports the view that this disease is widely prevalent as a subclinical infection.

Epidemiological evidence, therefore, consists of a series of interrelated facts from which a conclusion or series of conclusions may be drawn. The first step in epidemiological procedure is necessarily the demonstration of associations between the frequency of occurrence of the disease and some conditions or set of conditions; the second, the ascertaining of the relationship of these associations with one another; and third, their relations to the general epidemiological theory of the disease. To take a simple example, in milkborne typhoid fever it may be ascertained that cases of the disease occur predominantly along the route of the milkman, that in families so supplied the cases occur among those who drink milk, that the cases appear within a limited time to suggest simultaneous infection, and that an employee of the dairy is a carrier of typhoid bacilli. Not only are these associations related to one another but they are consistent with the general epidemiological theory of typhoid fever, viz., that transmission is basically a matter of the transfer of infected fecal material (or urine) from a patient or carrier to the mouth of a susceptible person.

While this would appear self-evident, there is an opinion of some prevalence that, since epidemiological evidence is necessarily purely circumstantial, it cannot be conclusive. This is perhaps due in large part to failure to appreciate the development, often basically statistical and therefore mathematical, of the logical analysis, and the significance of the body of evidence as a whole. The method of analysis and interpretation of epidemiological evidence is identical with that of experimental evidence, the practical difference lying in the fact that, as pointed out earlier, the epidemic is an experiment done for, rather than by, the observer and cannot be manipulated.

THE CONTROL OF INFECTIOUS DISEASE^{2, 35, 54}

With an understanding, albeit admittedly imperfect, of the factors which determine the means and extent of the dissemination of infectious disease, comes the possibility of control. The variety of epidemiological types of disease and the individual variability within these broad groups make for differences in control measures, and the control of a given disease under given circumstances almost always constitutes a special case. In general, however, control measures are of three kinds, *viz.*:

- (1) Those directed toward reduction or elimination of the source of infection, such as
 - (a) Quarantine and isolation of cases and carriers
 - (b) Destruction of an animal reservoir of infection(c) Treatment of sewage to reduce water contamination
 - (d) Therapy that reduces or eliminates infectivity of the individual
- (2) Those designed to break the connection between the source of infection and susceptible persons, *i.e.*, general sanitation measures such as

(a) Chlorination of water supplies

(b) Pasteurization of milk

- (c) Supervision and inspection of foods and food handlers
- (d) Destruction of insect vectors
- (3) Those that eliminate the susceptible and raise the general level of herd immunity by immunization, including

 (a) Passive immunization to give a temporary immunity following exposure or when a disease threatens to take epidemic form

(h) Active immunization to protect the individual from disease and the host population from epidemic disease.

One or another, or a combination of, such control measures are applicable to most infectious diseases of man and may be illustrated for the major epidemiological types of disease.

Diseases of lower animals transmissible to man.⁴³ Diseases in this group have in common an animal reservoir of infection. Direct control of the source of infection is, therefore, sometimes possible. When the transmission is direct, as in rabies, the reservoir of infection in the domestic dog may be controlled by immunization and quarantine of animals. The reservoir in wild animals, however, is not subject to such control, and complete elimination of the disease cannot be accomplished except locally under exceptionally favorable circumstances. The animal reservoir of bovine

tuberculosis is, of course, subject to direct control and may be, and has been in this country, eliminated by slaughter of infected animals. Here the pasteurization of milk also provides protection to the human population.

Insectborne diseases. Control of diseases transmitted by insect vectors is often most readily approached through control of the vector. Thus malaria control is, for all practical purposes, mosquito control, and louseborne typhus may be controlled by measures directed against the human body louse as well as by immunization of man. In instances such as these the vector is closely tied to man, by preference or by necessity, but when a disease has a reservoir of infection in wild animals and is transmitted by a vector that bites both those animals and man, control is more difficult. Plague is an example of an intermediate disease in this respect. The rat reservoir of infection is closely associated with man. and both the rat and rat flea populations are subject to an effective degree of control. but neither the wild rodent reservoir nor the insect vectors of sylvatic plague are subject to any appreciable degree of control. Similarly, tularemia and spotted fever, the latter having a reservoir in the tick, cannot be effectively controlled.

Diseases transmitted from man to man. Control of the source of infection in these diseases is a matter of quarantine and isolation, and in some instances the continued application of these may tend to eliminate the source. Thus, it is possible that human tuberculosis may eventually be practically eliminated in this country by isolation of cases; for many years the disease has not been reproducing itself, and the incidence shows a steady decline. Isolation is not, however, applicable to those diseases in which the reservoir of infection is the asymptomatic casual carrier, such as diphtheria. The diseases that are largely transmitted indirectly by water, food, milk, etc., are controlled with relative facility by general sanitation practices such as chlorination of water supplies and pasteurization of milk. In some cases these control measures also eliminate or reduce the reservoir of infection in a given area. Cholera, for example, has for many years been nonexistent in this country and western Europe. Typhoid fever is a more difficult matter because of the chronic carrier state, but when it is adequately controlled, the carrier incidence declines, thus reducing the reservoir of infection. As yet, airborne infection cannot be controlled so far as transmission is concerned.^{23, 24, 25}

When immunization is effective, these strictly human diseases can be controlled by its mass application, but mass immunization is weighed against other methods of control in terms of practicality. While, for example, a reasonable degree of immunity against typhoid fever may be obtained, chlorination of water supplies is a much more practical method of control of this disease than mass immunization under ordinary conditions in this country. In other diseases, however, such as diphtheria and small pox, mass immunization is by far the most practical method of control.

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Chapter Eleven

THE MICROBIOLOGY OF WATER AND SEWAGE

Of the interrelationships between the members of human populations that facilitate the transmission of infection, those arising as a consequence of common water supplies and the group disposal of sewage are among the most important. Transmission of disease by this means is confined to diseases of the so-called enteric group, in which infection takes place via the gastrointestinal tract and the causative microorganisms are discharged with the feces: Infection is, therefore, the result of a direct connection between infectious fecal material and the mouth of a susceptible person, and when that connecting link is a common water supply, as it frequently is, outbreaks of cholera, typhoid fever, and the like occur. This link is, however, readily broken; the great waterborne epidemics are rapidly becoming a thing of the past as a consequence of the utilization of effective control measures, and present-day waterborne epidemics are indicative, not of a lack but of a failure to make use of existing knowledge.

WATER11, 24, 26

Because of waterborne disease and the obvious desirability of its control, studies of the bacteriology of water have been directed, for the most part, toward its sanitary aspects. The best single criterion by which the sanitary quality of a water may be judged is, clearly, the kind and numbers of bacteria that are present in it. If it were possible, as a routine procedure, invariably to detect the presence of the appropriate disease-producing bacteria, it would be unnecessary, from

the sanitary point of view, to take the non-pathogenic forms into consideration. This is, however, not the case; a judgment of the sanitary quality of a water cannot be made on the basis of failure to find a microorganism such as the typhoid bacillus in that water. This bacterium, for example, is usually so outnumbered by similar forms such as the colon bacillus that its isolation and identification require enrichment cultures in selective mediums and other time-consuming procedures; even then these are not always successful, and a negative finding is of doubtful value.

It is legitimate to assume, however, that when a water is polluted with human fecal material, it is probable that it contains bacteria which cause enteric infection. This probability becomes a certainty when the fecal material is pooled, as in the sewage of a community, because of the ubiquitous presence of healthy carriers of typhoid bacilli and similar microorganisms. Since it is impractical to isolate these pathogenic bacteria themselves as a routine measure. some indicator of fecal pollution will serve equally well as a criterion of the sanitary quality of a water. The question, which was stated many years ago, becomes this: Are there types of bacteria which are never present in natural waters free of pathogenic bacteria but which may always be found in water polluted with human fecal material and therefore probably with pathogenic bacteria? If so, such types of bacteria can be used as indicators of pollution. The answer to this question involves a consideration of the bacterial flora of natural waters, including both bacteria which are native inhabitants of water and those microorganisms whose presence is a consequence of contamination from external sources.

Bacteria native to natural waters. The bacteria whose native habitat is water are not well known, in part because many of them are difficult to grow on laboratory mediums. There is no question, however, of the existence of a bacterial flora normal to and characteristic of natural waters. The types of bacteria which make up this natural population may be considered briefly:

- Higher bacteria, frequently the sheathed forms assigned to the Chlamydobacteriales and including sulfur, iron, and other forms
- (2) The Caulobacteria, a group of "stem" bacteria which occur in lakes and other bodies of water attached to some inanimate object
- (3) The spiral forms, which are frequently found in great numbers in water, some of which may be very large, 20 to 30 μ in length, as compared with the parasitic spirilla
- (4) A variety of bacilli, including
 - (a) Pigmented forms such as Serratia marcescens, Chromobacter violaceum, and Bact. aurescens
 - (b) Various nonpigmented forms such as:
 - 1. The fluorescent bacteria Pseudomonas fluorescens
 - 2. Certain of the sulfur bacteria
 - 3. Thermophiles
 - 4. Aerobic, spore-forming bacilli of uncertain taxonomic position
- (5) Coccus forms, both
 - (a) Pigmented, generally yellow—very often Sarcina lutea, and
 - (b) Nonpigmented-Micrococcus aquatilis, M. candicans, and others
- (6) Nitrogen-fixing bacteria—Azotobacter aquatile in particular
- (7) Nitrifying bacteria both Nitrosomonas and Nitrobacteria

These water bacteria are found in fresh water in swamps, streams, and lakes: The bacterial populations of salt waters have not been studied as extensively, but it appears⁴⁰ that the sea contains similar bacteria, including the nitrogen-fixing forms, the fluorescent bacteria, various pigmented forms, and the like. The water bacteria bear the same relation to the constant and recurring transformations of organic matter that take place in natural waters as they do to the same processes in the soil and are, therefore, an integral part of aquatic life. ¹⁰

Some, but by no means all, of the water bacteria can be cultivated on laboratory mediums. In any case no one medium suffices; the nitrifying bacteria, for example, cannot be grown in the presence of organic matter. Since the bacteria found on plates are of necessity only those that will grow upon the medium used, it will be clear that the value of plate counts as estimates of the numbers of these microorganisms is questionable. Enrichment cultures are sometimes useful, and it may be noted parenthetically that the simple holding of a water sample for a period of hours will frequently result in a marked increase in numbers. Considerable information has been obtained by a method of "slide culture" in which a clean glass slide is suspended in the water for several days, removed, stained, and studied microscopically.

Bacterial contamination of natural waters. In addition to the native water bacteria, a given water may, and usually does, contain a variety of bacteria as contamination from external sources. These sources are two—the air and soil, and human and animal excreta.

Bacteria from the air and soil. The number of bacteria in the air bears, as might be supposed, a close relation to the quantity of larger suspended particles or "dust." There are fewer bacteria in the air of the country than of the city, fewer in mountain air than in the air of the lowlands, and the air in mid-ocean is nearly bacteria-free. Under appropriate circumstances bacteria suspended in the air are those expelled from the upper respiratory tract of human beings and are of no small significance in the transmission of "droplet infection," but insofar as air in general is concerned this is an insignificant and relatively rare occurrence. Pathogenic microorganisms, such as the tubercle bacillus and the pyogenic cocci, have been found in the air of hospitals and sickrooms, but as a rule pathogenic bacteria in dry dust are exceedingly rare. The kinds of microorganisms in the air vary somewhat in different localities, but certain forms are generally present. Molds and yeasts are quite common and in some instances outnumber the bacteria. These include various species of molds such as Penicillium glaucum (the blue-green mold) and various yeasts, many of them the pigmented (red) torulae. Bacillus subtilis and related forms, together with the various micrococci, often pigmented, are almost universally present.9 They find their way into natural waters through settling out or are swept down by

Many of the bacteria present in the air, particularly the aerobic spore-formers, are essentially soil forms blown up in dust and able to survive drying. The soil itself contains tremendous numbers of bacteria; a gram of average field soil probably contains 100 million to 50 billion living bacteria, most of which are found in the upper 6 inches of soil, few being found in undisturbed soil below a depth of 4 to 5 feet. The great majority of these microorganisms are native to the soil and include the nitrogen-fixing and nitrifying bacteria, bacteria of the amylobacter group, and a variety of forms whose biochemical activities are an integral part of the mechanism of the decomposition of organic matter in the soil. A small part of the native bacterial flora of the soil consists of potential pathogens such as Clostridium tetani, Cl. edematis, and Cl. botulinium.

Bacteria other than those which make up the normal flora of the soil may be present as contamination. For practical purposes the pathogens that may be present may be regarded as coming from one of two sources: the flesh of animals and of persons who have died of infectious disease, and the excreta of human beings. In the first instance only one organism, the anthrax bacillus, is of significance, for other bacteria causing diseases of animals and man, such as the causative agents of tularemia, plague, spotted fever, and other diseases of animals, together with the bacteria of human disease such as the diphtheria bacillus and streptococci, do not survive long in the soil.

In the case of the anthrax bacillus, however, the spores are able to survive for long periods, perhaps years, and are brought to the surface from buried bodies by earthworms. Pastures may, then, remain infective for cattle for a long time.

The pathogenic bacteria contained in human excreta are those microorganisms which leave the body via the intestinal tract, *i.e.*, those causing the enteric diseases. The bacteria do not multiply in the soil, but their vitality may be considerably prolonged, possibly for two or three months.

It will be clear that microorganisms present in the air and in the soil have relatively ready access to bodies of water, and contamination may take place either more or less continuously or at irregular intervals under certain unusual conditions, as during and immediately after heavy rains. The contribution of air and soil, the latter in particular, to the bacterial flora of water is, then, considerable. Some of the potential pathogens, e.g., the bacilli of tetanus and malignant edema, are not infective when taken by mouth, and others, even though they survive for sufficient periods, rarely produce infection by this route. The normal portal of entry of the enteric bacteria is, however, the gastrointestinal tract, and it is these microorganisms which constitute the significant contribution of contamination through the agency of soil.

The intestinal microorganisms may be washed directly into lakes and rivers or other bodies of water by heavy rains; hence the presence of excreta on water sheds and consequent contamination of impounded waters is of considerable practical importance. In many instances, particularly in the case of shallow wells and the like, typhoid and other bacilli enter the water supply from privies, latrines, and similar devices via ground water. The distance over which such contamination can travel is a function of the rate of death of the bacteria and the rate of ground water flow. The latter is obviously dependent upon many factors such as the amount of rainfall, the local geological formations, and the like, and each instance of contamination, or possible contamination, must be considered individually. In general, fine soils and sand tend to impede their progress to a greater extent than coarse sand and gravel. The rock formations are of considerable significance; sandstone, for example, filters out bacteria, while limestone tends to erode with the formation of direct communicating channels, and wells drilled into such formations may contain typhoid bacilli which entered the water many miles away and are, therefore, always to be regarded as dangerous. The rapid spread of suburban living, often beyond municipal water supply and sewage disposal systems, has resulted in a revival of the problems of ground water contamination37 formerly confined to rural areas.

Contamination by excreta. Contamination of water by human excreta may take place, not only indirectly through the agency of soil as noted above, but also directly. Such direct contamination is, for the most part, a consequence of human population densities and urban organization and takes the form of dumping of sewage of one com-

munity into a body of water which serves as a water supply to another. Whether directly or indirectly contaminated, such waters contain not only the native bacterial flora supplemented by microorganisms from the soil, but also the flora of the human intestine. The contribution of the last consists of very large numbers of coliform bacilli. and much smaller numbers of Cl. welchii, the α -hemolytic fecal streptococci or enterococci (Streptococcus fecalis), together with whatever pathogens may be present. The pathogenic microorganisms are the pathogenic enteric bacteria such as the typhoid and paratyphoid (Salmonella); dysentery bacilli; where the disease is endemic epidemic, the cholera vibrio; certain of the animal parasites such as pathogenic amebae and Ascaris; and the so-called enteric viruses present in the bowel and excreted in the feces such as the polio and Coxsackie viruses and those of the ECHO group.

Under some conditions contamination by animal excreta assumes some importance. For example, surface water may contain coliform bacteria from domestic animals, and the presence of these does not carry the same implication as that of coliform bacteria of human origin. In other instances animal excreta may contain bacteria pathogenic for man, *viz.*, the contamination of water with leptospira from rat urine. Usually, however, contamination with animal excreta is not of great sanitary significance.

factors influencing the kinds and numbers of bacteria. The numbers of bacteria that may be found in a given water are dependent primarily upon the type of water, whether it is a surface water, such as that found in streams, lakes, and shallow wells, or a deep water from deep driven wells. In the first instance opportunities for contamination are great and, as might be expected, many bacteria are present. The water from deep wells, on the other hand, has undergone effective filtration in order to reach the deeper strata in which it is obviously not subject to any extensive contamination; hence relatively few bacteria are found.

A variety of environmental factors influences the bacterial content of water; chief among these are the amount of organic matter present and the temperature. In general, the more nutriment there is present in the form of organic matter, the greater is the number of bacteria. Low temperatures are not conducive to rapid growth and tend to keep the numbers of bacteria down, a factor that favors the survival of pathogens such as the typhoid bacillus which are unable to multiply in any case. Higher temperatures result in an increase in bacterial numbers in the presence of sufficient organic matter, but if the supply of nutriment is not great, after a preliminary increase during which the food supply is exhausted, the numbers fall below the initial level. Other environmental factors are not infrequently influential in determining the types of bacteria present in a water; thermophiles will, of course, predominate in hot springs and sulfur bacteria in sulfur springs, and the acidity of many natural waters results in a limited flora of acid-resistant bacteria.

Bacteria in ice. Although it is difficult if not impossible to sterilize a substance by exposure to low temperatures, many of the bacteria present are killed, only the resistant cells surviving. The great majority of bacteria in water are killed by freezing. Hence ice always contains but a fraction of the number in the water from which it was formed. Over 90 per cent both of the ordinary water bacteria and of typhoid bacilli die within a few hours, and a progressive decline in numbers then takes place, less than 1 per cent of typhoid bacilli surviving at the end of a week of freezing. Ice stored for six months is practically sterile. Outbreaks of typhoid fever have rarely been traced to the use of ice, although in a few instances the evidence of ice transmission seems quite conclusive. Danger of typhoid infection from the use of ice in drinking water is, in the absence of direct contamination of the ice during handling, always less than from the use of water from the same source as the ice.

The bacteriological analysis of water.^{1, 23} It will be apparent from the above discussion that the bacteria whose presence in water is a consequence of fecal pollution are not present in uncontaminated water and are sufficiently different from the native water bacteria that they may be readily distinguished. Of these bacteria the coliform bacilli are present in greatest numbers, while Str. fecalis and Cl. welchii, although constantly present, are usually not so numerous. It would appear, therefore, that any of these microorganisms could be used as an indicator of pollution. The coliform bacilli are

used almost exclusively in this country, and both *Str. fecalis* and *Cl. welchii* have been used in Europe. The streptococci are, however, sometimes difficult to differentiate and die out more rapidly than coliform bacteria. *Cl. welchii* has the disadvantage that its spores remain viable over long periods in contrast to coliform bacilli which, although more hardy than the typhoid bacillus, die out in time; hence *Cl. welchii* does not allow the differentiation of recent and old pollution.

The bacteriological examination of water for the presence of coliform bacilli rests upon the fact that this microorganism ferments lactose. The standard procedure for the examination has been prepared jointly by the American Public Health Association and the American Water Works Association and is revised at frequent intervals. Briefly, it consists of three parts: (1) the presumptive test, (2) the confirmed test, and (3) the completed test. In the first, lactose broth is inoculated with decimal dilutions of the water sample, commonly 10 ml., 1 ml., and 0.1 ml. (expressed as dilutions these are, respectively, 0.1, 1, and 10). The volume of the smallest inoculum producing fermentation provides a crude approximation of the numbers of coliform bacilli present in the water. Of the selective enrichment mediums containing ox bile and/or brilliant green or ricinoleate or lauryl sulfate, only lauryl sulfate tryptose broth has been officially accepted for the presumptive test without confirmation, and then not for filtered or treated waters. A more accurate estimate may be obtained by inoculating five tubes with each dilution and calculating the most probable number of coliform bacilli on the basis of the number of tubes in which fermentation occurs.30,36 Tables for calculation of the most probable numbers (MPN) are given by Prescott, Winslow, and McCrady.26 The confirmed test consists of the inoculation of a specified selective medium such as Endo or eosin-methylene blue (EMB) plates, brilliant green lactose bile broth, crystal violet lactose broth, fuchsin lactose broth, or formate ricinoleate broth. The appearance of typical coli colonies on the plates or fermentation in the selective lactose broth constitutes a positive confirmed test. In the completed test one or more typical colonies are picked from an Endo or EMB plate inoculated either from the original lactose broth culture or from the secondary selective medium showing fermentation and are transferred to an agar slant and a lactose fermentation tube. After incubation the slant culture is smeared and stained and examined for the gram-negative nonspore-forming rods of coliform bacilli. If the culture is found to be morphologically coliform bacilli and the lactose is fermented, the completed test is positive.

Membrane filter procedure.⁵ This method of enumerating bacteria in water, and in other fluids for special purposes such as quantitative blood culture, was introduced by Goetz in Germany in 1947 and subsequently was widely studied in this country. The microbial indicator is usually coliform bacilli, but enterococci may be enumerated also.²⁸

It consists essentially of the filtration of the water sample by negative pressure through a cellulose disc which will retain the bacteria,* removal of the filter disc to an absorbent pad† containing a differential liquid medium such as Endo medium in the case of coliform bacilli, and incubation. The bacterial colonies grow upon the surface of the filter disc and may be counted.

Duplicate 100 to 500 ml. volumes of finished, i.e., regarded as potable, water, single filtrations of each of 0.1, 1, and 10 ml. volumes of well water, and appropriately reduced amounts of polluted waters are examined. The volume to be filtered is not less than 20 ml., and when the sample is smaller than this it must be diluted to 30 ml. with sterile buffered water. The filter disc, sterilized in the autoclave prior to use, is removed after filtration and rolled, avoiding the entrapment of air bubbles, onto the absorbent pad containing nutrient medium in a sterile petri dish. The membrane is incubated for two hours and then transferred to a fresh pad saturated with the nutrient medium, and incubated overnight. The colonies are then counted.

The coliform counts so obtained are somewhat lower than those estimated by the MPN method. Whether the two methods do not measure precisely the same microorganisms, or whether the difference is a

^{*}Such as the Millipore type HA grid marked filters or their equivalent.

[†]Equivalent to Schleicher and Schuell No. 470 filter paper, approximately 48 mm. in diameter and capable of absorbing 1.8 to 2.2 ml. of nutrient medium.

result of the mathematical bias³³ of the MPN method, which is reported to give estimates 20 to 25 per cent higher than the actual coliform density, is not yet clear.

This is the direct membrane filter test. When the completed test procedures described above, *i.e.*, representative colonies, are examined further and shown to be nonspore-forming gram-negative bacilli which produce gas in lactose fermentation tubes, the test becomes the verified membrane filter test.

The microbial indicator. It is self-evident that a satisfactory indicator of pollution must persist at least as long as, and preferably longer than, the pathogenic microorganisms that may accompany it. The microorganisms used, coliform bacilli, enterococci, and Cl. welchii, all persist longer than the pathogenic enteric bacilli, such as the typhoid bacillus, and the first may under some circumstances show a transitory multiplication. The survival of enteric viruses is similar to that of pathogenic bacteria, but at least some of them, such as Coxsackie viruses, may persist longer than coliform bacilli at low temperatures, 8° to 10° C.,8 and these and other viruses such as poliovirus are more resistant to chlorination than coliform bacilli. In the case of treated waters, then, coliform counts may be reduced to "acceptable" levels even though some of the enteric viruses may persist. The matter of viral contamination of potable water has not yet been satisfactorily settled.

The casual and inclusive term coliform bacilli has been used advisedly above. The group is a heterogeneous one within the larger group of gram-negative enteric bacilli (Chap. Nineteen). Those which ferment lactose promptly are the microorganisms found in the foregoing tests for fecal pollution and include the coli and aerogenes types together with forms intermediate between them. These are placed in different genera, e.g., Escherichia coli and Aerobacter aerogenes in this country, and in the genus Bacterium elsewhere, and it is becoming apparent that A. aerogenes as currently defined is contracting, the great majority of these forms becoming A. cloacae or Cloaca cloacae, depending upon the nomenclature followed.

Aside from matters of nomenclature, differentiation of these coliforms from one

another is readily accomplished by biochemical reactions which are standardized for this purpose (Chap. Twenty). Whether such differentiation has any significance with respect to the sanitary quality of a water is another matter. It has been felt by some that the coli type is to be associated primarily with fecal contamination, while the aerogenes type may occur free in nature and that the latter has less sanitary significance. However, the aerogenes type also occurs in the bowel and is seldom found in the complete absence of possible fecal contamination of soils, and it is doubtful that the differentiation of the two types has significance in other than exceptional conditions.

As indicated above, the α -hemolytic streptococci of fecal origin, the enterococci. and Cl. welchii tend to be of uniquely fecal origin, and have been used as indicators of pollution. The enterococci have been of interest in this country in connection with the microbiological assay of swimming pool waters (see below), though here also the coliform bacilli have been used most often. In general, a lack of suitable culture mediums for the detection and enumeration of enterococci has been a practical difficulty, and a number of reasonably satisfactory culture mediums have been developed.22, 29 Such mediums are made selective by the inclusion of sodium azide, and differential with certain dyes such as ethyl violet or triphenyl tetrazolium chloride, and the membrane filter method may be applied.²⁸ Enterococci tend to persist longer than pathogenic enteric bacteria, but not as long as coliforms, and are usually found in considerably smaller numbers than coliforms.

Plate counts. It is usually desirable to have an approximate measure of the total number of bacteria in drinking water, not because the sanitary quality of a water can be judged on this basis alone, but because such information frequently has ancillary value. The counts obtained by quantitative dilution and plating are, it must be remembered, those of the microorganisms that will grow on the medium used, other bacteria being quite inapparent by this method.

Two series of plates are poured, one in which the medium is nutrient gelatin and the other nutrient agar, or both may be nutrient agar. The gelatin plates, or one set

of agar plates, are incubated at 20° C. and the agar plates at 37° C. In general the native water and soil bacteria grow best at 20° C., in some cases they do not grow at all at 37° C., and bacteria of animal origin grow most rapidly at body temperature. The relative numbers of microorganisms growing at the two temperatures are, then, at times suggestive of the origin of the bacteria found.

Chemical analysis. The analysis for appropriate chemical compounds frequently is of value as an adjunct to bacteriological analysis in the determination of the sanitary quality of a water.1, 23 Pollution by sewage, for example, adds complex compounds protein, carbohydrate, and fat – to the water, and the amount and state of the decomposition products of these substances may serve as an index of the degree and time of pollution. Ammonia, nitrites, nitrates, chlorides, and albuminoid nitrogen are usually determined. Of these, chloride and, to a certain extent, nitrate, are the most useful. Chemical analysis of water is frequently made in connection with hardness, turbidity, taste, smell, and similar features, which, while often of considerable industrial or esthetic significance, are of no sanitary importance.

The assay of the sanitary quality of water.¹ The means by which the sanitary quality of a water is judged may be summarized briefly:

- (1) The bacteriological analysis, including both
 - (a) The presence or absence of coliform bacteria and
 - (b) The number and type of bacteria present
- (2) The type of water, whether surface or deep
- (3) The local conditions
- (4) Chemical analysis.

Of these the presence and numbers of coliform bacteria are the most important, and it must be remembered that the relative abundance, rather than the presence, of these microorganisms is the essential feature of the test. The discovery of a single colon bacillus in 50 ml. of water, or even occasionally in 5 ml., affords no reasonable ground for suspicion of the water. The possibility of sporadic contamination with colon bacilli derived not from man but from domestic animals or birds must be kept in mind. Manured fields and pastures, filled with grazing cattle or sheep, are likely sources of colon bacilli and may give rise to mistaken inferences if the environmental examination of a water supply is neglected. Knowledge of such local conditions as well as the type of water is, then, essential to the interpretation of the bacteriological findings. Chemical analysis may be of considerable help in some instances, but in general finds its greatest utility in the study and control of gross pollution in which the decomposition of organic matter and presence of industrial wastes are a nuisance, rather than the assay of the purely sanitary quality of a water.

Drinking waters. The question of the significance of the bacteriological findings brings up the matter of standards to which a water suitable for drinking should conform. Now it will be clear that, although considerable numbers of colon bacilli in a water are always suggestive of fecal contamination by man or animals, a standard is necessarily a minimum which is inherently difficult to define under the circumstances. In this country the United States Public Health Service has prepared recommended standards³⁴ which are intended to represent a minimum. In the recommended procedure the frequency of sampling is dependent upon local conditions, the minimum varying from one sampling per month for a population of 2500, to 500 per month for a population of five million. The sample is five 10 ml. portions, or five 100 ml. portions. The results of bacteriological examination by Standard Methods procedures should be as follows:

- Of all 10 ml. portions examined per month, not more than 10 per cent shall show the presence of coliform bacteria.
- (2) Occasionally more than three of the five samples show coliforms; this must not occur in more than 5 per cent of samples when 20 or more samples are taken per month, or in more than one sample if less than 20 samples are taken per month.
- (3) Should such a result (as in 2) be obtained from a single standard sample, daily testing must be carried out until at least two consecutive satisfactory samples have been found. Such daily samples are to be regarded as "special samples" and not included in the monthly totals.
- (4) With regard to the 100 ml. samples:
 - (a) Not more than 60 per cent shall show coliforms.
 - (b) Occasionally all five portions constituting a single sample will show coliforms; this must not occur in more than 20 per cent of samples when five or more samples are examined per month, or in more than one if less than five samples are taken per month. If so, daily "special samples" must be taken as above.

The water shall be satisfactory as to taste, odor, and color and shall contain less than the following minimum quantities of chemical impurities: lead, 0.1 ppm; fluo-

rine, 1.5 ppm; arsenic and selenium, 0.05 ppm; copper, 0.3 ppm; iron and manganese, 0.3 ppm; magnesium, 125 ppm; zinc, 15 ppm; chloride and sulfate, 250 ppm; total solids, 500 ppm; phenol, 0.001 ppm.

The British Ministry of Health suggests standards²³ based on the presumptive coliform count as determined by acid and gas formation in MacConkey broth on the piped supply entering the distribution system. Waters are divided into classes on the following basis:

Class I—Water of class I and regarded as highly satisfactory contains less than one coliform per 100 ml.

Class II—Water regarded as satisfactory contains one to two coliforms per 100 ml.

Class III—Water regarded as suspicious contains three to 10 coliforms per 100 ml.

Class IV—Water regarded as unsatisfactory contains more than 10 coliforms per 100 ml.

While a presumptive test alone is specified, the occurrence of positive presumptive tests has been found to be very high in Britain. Waters of classes I and II conform closely to the American standards.

The World Health Organization has compiled standards in the sense of bringing together common practices and criteria of water examination, together with suggested procedures;³⁸ in general these resemble closely those described above.

Swimming pools and bathing places.²¹ The sanitary control of water in swimming pools and bathing beaches is similarly based on bacteriological examination. The American Public Health Association has recommended that not more than 15 per cent of samples of swimming pool water contain more than 200 bacteria per milliliter or give positive confirmed tests for coliforms in any of five 10 ml. samples when the pool is in use. The standard is as high as that for drinking water, but pools are usually filled with water of drinking quality, and pollution is not only derived from bathers but is fresh and may be highly infective. The presence of acid-forming streptococci is also of considerable utility as a measure of oral and skin contamination, and usually corresponds closely to the total count. Staphylococci, present in the nose and throat and on the skin, would appear to be a logical indicator for swimming pool water, and their use has been proposed.6 It has been shown that the number of staphylococci is directly related to the bathing load, and being more resistant they persist longer than coliforms, so that relatively large numbers of staphylococci may be present in the virtual absence of coliforms.

Such waters commonly contain residual chlorine which must be neutralized with thiosulfate when samples are collected for bacteriological examination. In natural outdoor bathing places the test for coliforms is the most important. The standards are necessarily much more lenient than those for indoor pools, and those adopted locally vary from an allowable 100 coliforms per 100 ml. in California and Indiana to 3000 coliforms per 100 ml. allowed by the New York City Health Department.

Purification of water supplies. 13, 39 When, by bacteriological examination or otherwise, a water is known to be unsafe for consumption, the question arises as to ways and means of artificial purification. There are a number of useful methods of purifying water, differing according to the amount and character of the water to be treated, which may be summarized as follows:

- (1) Mechanical methods
 - (a) Storage
 - (b) Filtration
 - 1. Slow sand filtration
 - 2. Coagulation and rapid sand filtration
- (2) Chemical methods
 - (a) Large scale-hypochlorites and liquid chlorine
 - (b) Small scale—hypochlorite, ultraviolet light, ozone, etc.

Of these, storage is not generally regarded as a method of water purification, though the numbers of bacteria are usually greatly reduced in impounded waters because of the exhaustion of the food supply and the consequent death of bacteria and settling, not so much of bacteria alone as of suspended matter which carries down bacteria with it. The partial removal of suspended matter is frequently desirable, particularly with turbid waters, and may be carried out by allowing the water to remain in a settling basin for a time.

Slow sand filtration, one of the earliest and most effective methods of water purification, is in use in many European and some of the older American cities. These sand filters are constructed so that the water passes through 1 to 5 feet of sand supported upon graded layers of gravel. The rate of filtration must be accurately regulated and

the efficiency of operation controlled by frequent bacterial test of the effluent. Such filters are highly effective. Bacteria are removed, not to any great extent by mechanical straining out, but through a biological mechanism in which the activity of protozoa is an important feature. The passage of water through these filters is necessarily a relatively slow process and, in consequence, relatively large areas are required, which, for financial or other reasons, are no longer available in large American cities. Few of these filters have been constructed in recent years, and the use of rapid sand filters is becoming common.

The rapid sand filters, which may be used with turbid waters that would clog a slow sand filter, are frequently employed in conjunction with "coagulation," the addition of such substances as aluminum or ferric sulfate, which form flocculent precipitates (the hydroxides). The precipitate carries down most of the suspended matter and, of course, many bacteria, and is readily filtered out, yielding a clear effluent containing relatively few bacteria. Such filters must be cleaned from time to time, a process that is seldom required with slow sand filters. They make possible, however, the treatment of a large quantity of water on a relatively small filtering area.

Destruction of pathogenic bacteria in water supplies is most often brought about by treatment with germicidal chemicals. Of these, hypochlorite (calcium hypochlorite or bleaching powder) was at one time used widely in the treatment of municipal water supplies, and it still has its uses, although it has been largely superseded in large-scale treatment by liquid chlorine, which is supplied in cylinders under pressure. Chlorine is added directly to the water through an automatic feeding device in accurately measured amounts which are determined by the character of the water. In general, the greater the amount of extraneous organic matter present, the greater must be the amount of chlorine added.

The amount of chlorine taken up is termed the chlorine demand, and the point at which the residual chlorine or available chlorine becomes proportional to the added chlorine is called the "breakpoint" in the chlorine demand curve. The introduction of breakpoint chlorination usually results in material improvement in the sanitary quality of a water. When liquid chlorine is added to ordinary surface water, clear and not highly contaminated, in the proportion of about 0.5 to 1 part of "available chlorine" per million gallons, the ordinary intestinal bacteria are destroyed, including such pathogenic forms as the typhoid bacillus. Certain of the enteric viruses, such as poliovirus, are more resistant to chlorination than are coliform bacilli, requiring a concentration of more than 0.2 ppm, while a dose as low as 0.1 ppm may be sufficient to destroy the coliforms. The question of the persistence of enteric viruses in treated waters has been pointed up by the occurrence of waterborne epidemics of infectious hepatitis, and has not yet been fully clarified. 14, 16, 18, 27, 32

The tastes and odors in chlorinated waters may be due to an overdose of chlorine caused by inadequate control methods or to the action of chlorine upon compounds present in the water, commonly as industrial wastes. Excess chlorine and tastes caused by chlorination can often be removed by dechlorination with SO₂ or treatment with KMnO₄ or activated carbon. The bactericidal activity of chlorine may be prolonged, particularly in waters containing considerable organic matter, by the simultaneous addition of liquid ammonia with the formation of chloramines.

The introduction of chlorination of municipal water supplies has, in practically every instance, resulted in marked reductions in the incidence of enteric infection, typhoid fever in particular, in this country, but the addition of chlorine or other bactericidal agents is not a cure-all. When a water is sufficiently heavily contaminated, bacteria embedded in particles of organic matter are not killed even in the presence of available chlorine. It is generally agreed that a content of more than 50 coliform bacteria per milliliter indicates pollution too great for successful chlorination.

Chlorine dioxide (ClO₂) also has been applied to the treatment of water. It has the advantages that it destroys algal tastes as well as the chlorophenol taste of certain chlorinated waters and oxidizes organic matter more rapidly than chlorine, thus allowing the maintenance of a chlorine residual in the distribution system. In water solution this substance is decomposed by light to chloric and perchloric acids and

319

oxygen, and is more bactericidal for coliform bacteria than chlorine.

Frequently a combination of filtration and chlorination is desirable, particularly with rapid sand filters. Not only does a preliminary filtration have esthetically desirable features in the cases of turbid waters, but considerably less chlorine is required for treatment than would have been the case had not the greater part of the suspended material been removed.

The chemical treatment of water on a small scale does not always involve expense as a primary consideration, and hence other methods may be used. The treatment of swimming pool water by ultraviolet irradiation adds no tastes or odor to the water and, although considerably more expensive than chlorination, can be used because the scale of operation is small.³¹ Ozone, which is strongly bactericidal, is also relatively expensive and is not commonly used in this country but is used to an appreciable extent in Europe. Chlorination is always available and bromine and iodine have been used also.²⁰

At times the treatment of water becomes an individual matter, as in the case of an army in the field or when a public supply is known to be impure. In the first instance the water may be treated by the addition of hypochlorite in the form of bleaching powder or a solution of sodium hypochlorite or two drops of a 2 per cent tincture of iodine per quart. Hypochlorite tablets and solutions are available for this purpose, and iodine compounds, such as tetraglycine potassium triiodide, have also been made into tablets under trade names such as Globaline, Potable Aqua, and Individual Water Purification Tablets. The last are said to be effective against ameba cycts, cercariae, and certain of the viruses as well as against the common pathogenic enteric bacilli.² In the home, filters, such as the Berkefeld or Chamberland bougies, may be used, but filtration is slow, and care in operation and frequent cleaning are necessary. The simplest and best method of water treatment for the family or individual, however, is simple boiling. Boiling for five minutes is quite sufficient to destroy with certainty the typhoid bacillus and allied forms as well as the cholera vibrio. When waterborne disease is prevalent, or when a water supply is notoriously impure or exposed to chance of infection, boiling is the only wholly safe procedure.

SEWAGE7, 19

Sewage is best regarded as the used water supply of a community and as such is a dilute solution of fecal matter and other wastes. From the hygienic point of view it is an important vehicle in the transmission of enteric infection; hence the manner of its disposal is of considerable significance. The mechanisms of sewage disposal have as their objects: first, the ridding of a community of an ever-present volume of waste and, second, disposal in such a manner that it is not dangerous to other communities.

The complex organic compounds present in sewage undergo the same processes of decomposition that are involved in the breakdown of dead organic matter in nature and are a part of the so-called cycles of elements such as nitrogen, phosphorus, and the like. Any type of sewage treatment, then, is nothing more than a mechanism for bringing about or accelerating these transformations. The organic compounds are first broken down to amino acids, monosaccharides, and the like, which are eventually oxidized completely to carbon dioxide and water in the case of carbon and hydrogen, and to nitrite and nitrate in the case of nitrogen. Bacteria are the active agents in this decomposition and oxidation, the mechanisms of which have been discussed earlier (Chap. Five). Although essentially simple in principle, sewage treatment is in practice a complex problem which cannot be considered at length here.4, 25

In general, however, sewage disposal falls into one of three categories: (1) dilution, (2) partial treatment, and (3) complete treatment. In the first instance the sewage is simply dumped into some body of water where it will not annoy its originators. Here the breakdown and oxidation of the constituents of the sewage occur in nature, and, if sufficient time elapses, no trace remains beyond an increase in nitrate which, in turn, serves as food material for phytoplankton. When this transformation takes place in a flowing stream, the phenomenon is known as the self-purification of streams. The

essential element, however, is not the fact that the water is in movement but that sufficient time elapses for the breakdown and oxidations to proceed to completion. Soil polluted with sewage similarly "purifies itself."

With increasing population densities the disposal of sewage by dilution becomes unsatisfactory because a body of water is not infrequently a water supply to a neighboring community. Some form of treatment, then, becomes obligatory; in other words, the decomposition is made to take place in whole or in part in the various tanks and other devices making up a sewage treatment plant rather than allowing it to occur in natural bodies of water. In practice, treatment takes the form of preparatory processes followed by a period of anaerobic digestion, and then a period of aerobic oxidation. Treatment may be complete even through nitrification, or the partially treated sewage may be disposed of by dilution.

When the disposal of fecal material is an individual or family problem the mechanics involved are somewhat different, but the processes of decomposition and oxidation are essentially the same whether a privy, cesspool, or septic tank is used.³⁵

It will be noted that, although sewage treatment is basically a bacteriological process, the pathogenic bacteria are not involved; their presence is not taken into consideration, nor is there any effort to destroy them except in the rare instances in which the effluent from a complete treatment plant is chlorinated. In one sense, then, sewage treatment is not directed toward the control of waterborne disease. The fact that it is treated, however, is of considerable significance in this connection. When sewage is disposed of by dilution, the typhoid bacillus and related microorganisms, together with enteric viruses, do not multiply; rather, a decline in numbers sets in immediately. and these bacteria do not survive as long in water as in soil, probably not more than a few days or a week and they tend to disappear during sewage treatment, although there is no assurance that pathogens are absent in the effluent from treatment plants.3, 12, 15, 17 Clearly then, fresh pollution is more dangerous than past pollution, and, in a sense, treatment obviates the possibility of such fresh pollution. The economic and esthetic aspects of sewage treatment are, of course, another matter.

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Chapter Twelve

THE MICROBIOLOGY OF MILK AND FOOD

Other than the ramifications of a common water supply, the most common vectors of disease of microbial etiology are the wide variety of foods consumed by man. Like

water, these constitute a link between the susceptible individual and the source of infection.

Milk ^{23, 27, 43}

As a vector of infectious disease, milk differs from water in that it is an excellent medium for the growth of many pathogenic bacteria, and from other foods in that it is the only food of animal origin that is consumed in large part in the raw state. Since large quantities of milk are consumed—it is estimated that about 16 per cent of the average dietary in the United States consists of milk and milk products—the importance of this substance in the transmission of disease is evident. The diseases transmitted by milk are: first, diseases of cattle transmissible to man and including bovine tuberculosis, undulant fever, foot-and-mouth disease, and streptococcal infections from infected udders and, second, diseases of man in which the milk serves as the link between man and man, such as typhoid fever, diphtheria, and rarely, certain other diseases such as poliomyelitis.

Sources of bacteria in milk. Unlike water, milk has no native bacterial flora, and it is probable that milk as secreted into the udder of a healthy cow is sterile. The milk in the udder is, however, rarely if ever bacteriologically sterile, for the microorganisms invade the udder via the milk ducts of the teats and the first portion of the milk drawn (foremilk) always contains more bacteria than the last (strippings). There is, further-

more, no bacterial flora that is characteristic of milk; the presence of microorganisms is always a consequence of contamination, and the types of bacteria found are determined by the source of contamination. From this point of view the bacteria of milk fall into two groups: first, those which are present in the tissues of an infected cow and find their way into the udder and, second, those which enter the milk, usually after it is drawn, from sources external to the animal.

Bacteria from infected cattle. Of these microorganisms perhaps the most important is the tubercle bacillus. These bacteria get into the milk directly as a consequence of tuberculosis of the udder, which occurs in 1 to 2 per cent of infected cows, and indirectly by contamination with cow manure when the infectious sputum is swallowed and discharged with the feces. In either case the milk is infective for man, the bovine variety of the tubercle bacillus giving rise, as a rule, to bone and joint rather than pulmonary tuberculosis. Milk may, of course, be infected with human tubercle bacilli from infected persons. A high proportion of the former type of infection occurs when the transmission of this disease is allowed to occur. The disease may be controlled at its source, i.e, by the elimination of infected cattle from dairy herds, a practice which is practically universal in the United States.

A bovine variety of *Brucella melitensis* (the causative agent of undulant fever), *Br. abortus*, infects cattle, producing the disease contagious abortion. This microorganism is excreted in the milk and infects man, producing a mild type of undulant fever. The caprine variety of this bacterium which is present in the milk of infected goats produces a much more severe disease in man. Undulant fever, like tuberculosis, may be controlled by control of the disease in the animal reservoir of infection.

The virus of foot-and-mouth disease, a virus disease of cattle, is excreted in the milk and is thus transmitted to man. The disease in man is mild, however, and not of great public health importance. More important are the streptococcal infections of the udder, designated garget or mastitis. The Hotis test is commonly used for the detection of mastitis; it consists of incubating fresh milk in the presence of 0.025 per cent bromcresol purple for 24 hours at 37° C. A positive reaction, the formation of yellow flakes on the side of the test tube, is dependent upon the presence of the streptococci and of agglutinins in the milk; the growing bacteria are clumped by the antibody and the acid reaction results from the fermentation of lactose. The streptococci of bovine mastitis, Str. agalactiae, are rarely found to be a cause of human disease, but milkborne epidemics of streptococcal infection may be associated with acute udder inflammation in the dairy herd, and the massive and continuous infection occurring in some of the outbreaks of human streptococcal infection indicates that the udders of the cattle were infected. It has been suggested that Str. pyogenes may proliferate, with or without symptoms, in the udder, and epidemics of human streptococcal infection may occur, though it is probable that in most instances milkborne infection is a consequence of direct human contamination. The udder may be infected occasionally with diphtheria bacilli, which produce small external ulcers, but this is an uncommon occurrence.

Bacteria from external sources. When milk is collected under ordinary conditions, the udder bacteria form but an insignificant fraction of the total number of microorgan-

isms in the milk. The skin of the cow, the hands of the milker, the vessels used for collection, and the dust of the cow barn all contribute their quota to the number of bacteria found immediately after milking. If milk is obtained with aseptic precautions, it contains only a few hundred (200 to 400) bacteria per milliliter; collected with somewhat less care, it may contain a few thousand (2000 to 6000); with careless manipulation, even freshly drawn milk may be highly contaminated (30,000 to 100,000 per milliliter). If milk is kept at 0° C. (32° F.), it shows a decrease in the bacterial content during the first few hours, but at higher temperatures the rate of multiplication is high and, when richly seeded at the outset, enormous numbers of bacteria result.

The nonpathogenic bacteria that are present in milk are often differentiated on a physiological basis into the following groups:

- (1) The acid-forming bacteria
- (2) The alkali-forming bacteria
- (3) The proteolytic bacteria
- (4) The inert bacteria

The first group includes the fermentative bacteria, and the most common type of fermentation is the lactic acid fermentation, the process by which milk usually sours under natural conditions. A variety of bacteria may be responsible, among them Staphylococcus aureus, Str. pyogenes, and Escherichia coli. A few species, however, are commonly active in the natural souring of milk, and these may be divided into two groups. One of these is comprised of the capsulated gas-forming bacilli of the Bacterium (lactis) aerogenes type, which are closely related to E. coli, differing principally in their possession of capsules, lack of motility, and ability to produce gas from potato starch. The second type, a streptococcus, Streptococcus lacticus (or lactis), is abundant in naturally soured milk, particularly when the acidity is high.

Lactic acid milks, regarded by some as having therapeutic value in the treatment of certain intestinal disorders, have been prepared by the inoculation of Lactobacillus species such as *L. acidophilus* and *L. bulgaricus*. The lactobacilli are commonly found in the fermentation of ensilage and are present in small numbers in the human mouth and intestinal tract and are not ordi-

narily responsible for the natural souring of milk.

Although lactic acid is commonly the predominating acid in the fermentation of milk, the formation of butyric acid is observed occasionally. This is generally a consequence of the presence of the anaerobic butyric acid bacteria but may be brought about by *Bacillus subtilis* and related sporeforming aerobic bacilli.

The spontaneous alcoholic fermentation of milk is less usual under natural conditions than either the lactic or the butyric, and the preparation of certain alcoholic beverages is dependent upon the artificial production of this form of milk fermentation. Koumiss, a drink prepared by Tartars by the alcoholic fermentation of mare's milk, and kefir, an effervescent sour milk prepared by inhabitants of the Caucasus from the milk of cows, goats, and sheep, are prepared by the inoculation of fresh milk with old koumiss in the first instance and with "kefir granules" in the second. The bacteriology of the koumiss fermentation is not well known, but in the case of kefir both a bacterium and a yeast appear to be involved. Some species of yeast are able to effect the alcoholic fermentation of milk in pure culture.

The alkali-forming bacteria are those organisms which do not ferment lactose but, presumably, act upon the nitrogenous substances present with the liberation of ammonia. When typhoid and paratyphoid bacilli, for example, are cultivated in litmus milk no effect is apparent beyond a slowly increasing alkalinity. Certain other bacteria, such as some of the aerobic spore-forming types, also produce lipase and decompose the fats present, converting the milk to a yellow transparent fluid.

The proteolytic bacteria also produce an alkaline reaction and, in addition, hydrolysis of the milk protein. Two enzymes or groups of enzymes, are responsible; one, a rennin-like enzyme, precipitates the protein with the formation of a soft curd, and a second, casease, brings about hydrolysis of the protein which, when complete, results in the conversion of the milk to a clear fluid, a process sometimes termed peptonization. Peptonization, however, does not follow precipitation when the microorganism does not possess casease. The bacteria producing these changes include spore-

forming aerobes such as *B. subtilis*, certain strains of staphylococci, *Proteus vulgaris*, and others. Proteolysis may occur in heated milk in which the nonspore-forming lactic fermenters have been destroyed, leaving the more resistant spore-forming proteolytic forms.

The "diseases" of milk. A series of unusual or abnormal changes, called "diseases" of milk, sometimes occur. "Blue milk," "red milk," and "yellow milk" are caused by the presence of various chromogenic bacteria. "Bitter milk," characterized by a bitterness that sometimes develops after a short interval, is likewise due to the products of certain microorganisms. Milk sometimes suffers from a ropy or slimy fermentation which, under most circumstances, is regarded as undesirable, although such a fermentation is intentionally produced in the manufacture of Edam cheese in Holland through the action of a particular species of streptococcus.

The inert bacteria are those which produce no visible change in milk. These include certain nonpigment-forming bacteria from water and other sources, and, in addition, most of the pathogenic bacteria found in milk. Such dangerous contamination, then, is inapparent without bacteriological examination.

The pathogenic bacteria from external sources.40 The multiplicity of sources of contamination results in the heterogeneous bacterial flora that may be found in milk. It is important from the hygienic view that, in addition to microorganisms of soil and water, the bacteria carried by man have relatively ready access to milk. The contamination with the bacteria of human disease is a constant possibility, not only when the milk is drawn but throughout its handling until it reaches the ultimate consumer. Contamination may be direct, as in the case of streptococci or diphtheria bacilli from the throat and typhoid bacilli from the hands of infected individuals, or it may be indirect, as when the water used to wash the milk cans is contaminated with typhoid bacilli. In any case, the microorganisms do not simply survive, as in water, but actively multiply and may be present in huge numbers in pooled milk of which but a single part was originally con-

Clearly, then, the most important factors

governing the number of bacteria which may be present in milk are, first, the kind and degree of initial contamination and, second, the temperature at which the milk is kept. The production of hygienically satisfactory milk, therefore, involves cleanliness in the first instance, and immediate cooling and storage at a low temperature in the second.

The bactericidal property of fresh milk. As indicated above, the number of bacteria present in freshly drawn milk frequently shows an initial decrease. Its extent may be exaggerated by plate counts; the various antibodies are present in the milk, not only the bactericidal substances but the agglutinins as well, which, by clumping the bacteria, may bring about decreased plate counts. That freshly drawn milk is both bactericidal and bacteriostatic, although to a mild degree, is, however, definitely established. This activity is thermolabile. being destroyed in 15 minutes at 75° C. and in two minutes at 80° to 90° C., and disappears a few hours after the milk has been drawn.

The determination of the quality of milk. By far the best index of the quality of milk is the number of bacteria it contains. The bacteria present are, in all cases, a result of contamination, and hence these microorganisms are reliable indicators of cleanliness and care and are generally used for this purpose.

Plate counts. The total bacterial count of a milk is a reflection of its hygienic quality, and the plate count has been, and still is, widely used in the bacteriological grading of milk. Standardization of the mediums and of the technique is necessary for com-

parable results. A standard procedure has been developed under the auspices of the American Public Health Association.²

Standards of milk quality in terms of plate counts are in general use but vary from one locality to another. In some instances more than one grade of milk is allowable; for example, a "Grade A" milk may contain not more than 30,000 bacteria per milliliter as delivered, and a "Grade B" milk not more than 100,000. There is a steady movement toward more strict reguirements, and the maximum number of bacteria allowed is continually reduced. More than one grade of milk is undesirable in that the distribution of inferior milk is allowed, and, when possible, a single standard is preferable. In Chicago, for instance, there is but one grade of milk, and it must not contain more than 10,000 bacteria per milliliter as delivered to the consumer.

The membrane filter technique used for the bacteriological examination of water (Chap. Eleven) is not useful for the routine examination of milk. It is not possible to pass significant quantities of milk through the filter disc without clogging, and if this method is applied the bacteria must first be removed by centrifugation and filtered after resuspension in buffer or saline. It may be applied to equipment rinse solutions, etc.

Microscopic counts. Besides the ordinary macroscopic colony count obtained by plating, a microscopic method devised by Breed is of value for many purposes. In the Breed method milk is taken in a capillary pipette discharging 0.01 ml. and is dried over an area of 1 sq. cm. on a glass slide. After washing out the fat with xylol and fixing with alcohol, the film is stained with

Milkborne Epidemics of Disease*

DISEASE	OUTBREAKS		CASES	
	NUMBER	PER CENT OF TOTAL	NUMBER	PER CENT OF TOTAL
Typhoid and paratyphoid fever	76	45.2	1,209	12.1
Septic sore throat and scarlet fever	57	34.0	6,812	68.2
"Gastroenteritis"	24	14.3	1,423	14.2
Bacillary dysentery	5	3.0	411	4.1
Diphtheria	5	3.0	123	1.2
Poliomyelitis	1	0.6	11	1.0
Totals	168	100.0	9,989	100.0

^{*}In New York State exclusive of New York City as reported by Dublin, Rogers, Perkins, and Graves. 16

methylene blue. The number of bacteria per square centimeter is estimated by counting a measured area. The ratio used in comparing the microscopic count with the standard plate count is 4:1, although it is recognized as inaccurate and variable. This direct microscopic count does not involve incubation and has proved of special value in judging the quality of fresh milk as delivered to milk-receiving stations.

Methylene blue reduction.34 If a small amount of methylene blue is added to milk and the mixture incubated, the dye will, in time, be reduced to the colorless leuco base. Although freshly drawn milk has some small power to reduce this and other dves. the reduction is, for all practical purposes, a consequence of the metabolic activities of contained bacteria. There is, then, a direct relation between the time required for reduction and the number of bacteria present, and the measurement of this reduction time provides a useful approximation of the quality of a milk. The method is best adapted to raw milk and has the great advantage that no special apparatus or training of the operator is required. Although milk cannot be graded with any degree of precision in this way, it is generally agreed that a milk decolorizing in less than two hours is poor in quality, while that which does not decolorize in eight hours is excellent. Resazurin has been used to some extent in the place of methylene blue.

Coliform count. The coliform index of water pollution is not generally applicable to milk. Water contamination of sanitary significance is fecal, but this is not the case with milk; coliform bacilli, for example, would not be associated with the presence of the diphtheria bacillus in milk. Further, since the great majority of market milks contain cow feces, the estimates varying from 60 to 100 per cent, coliform bacilli in milk are probably most often of bovine origin. Although the coli count has been used widely in the past, it is not worth while except under special circumstances.

The isolation of pathogenic bacteria. In contrast to water, the isolation of pathogenic bacteria from milk is not only a practical but often a desirable procedure. The presence of tubercle bacilli, for example, can be conclusively shown only through their isolation, in this instance usually by guinea pig inoculation. Other pathogens,

such as hemolytic streptococci and Brucella, may be isolated by culture. The isolation of these bacteria is not a routine procedure but may be carried out when an outbreak of disease is suspected of being milkborne.

Cell count and sediment test. The number of leucocytes present in a sample of milk and the amount of contained dirt that can be strained out through a standard cotton disc are frequently of value in the assay of milk quality. Excessive numbers of leucocytes are present in the milk from infected udders, and when their presence is noted in Breed smears, mastitis is suggested. The amount of sediment that a sample of milk contains is an index of the extent to which it has been contaminated and is often, though not necessarily always, correlated with the bacterial count.

The hygienic control of milk.⁴⁶ The sanitary quality of milk may be controlled in one or both of two ways: in the first instance, by preventing to a considerable degree the contamination of milk and the multiplication of contained bacteria and, in the second, by destruction of bacteria, the pathogenic forms in particular, already present in the milk.

Inspection. The periodic inspection of dairy farms is carried out by the local board of health in many places and, although it does not insure the absence of pathogenic bacteria from milk, it is effective in increasing cleanliness and reducing the numbers of bacteria. A score card is frequently used, and a given dairy farm is rated according to a system of points.

Certified milk. One of the earliest attempts to avoid the dangers of milkborne infection was the elaboration of methods designed to safeguard milk at every step in its production, collection, and distribution. To this end "Medical Milk Commissions" were established in a number of localities in the United States, usually under the auspices of the local medical society. Milk conforming to certain standards is certified by such a commission to be of high quality. The regulations, which are generally excellent, deal with such matters as the cleanliness of barnyard and dairy, the purity of the farm water supply, the proper sterilization of utensils, and the health of the cows and of the milkers. The bacterial content is limited to 10,000 per milliliter, and the milk must be delivered within 36 hours.

Certified milk is undoubtedly safer to use than milk collected and transported without suitable supervision, and the work of the milk commissions did much to improve dairy conditions in many parts of the country. At the same time, raw milk, certified or not, can never be regarded as protected against all chances of contamination; the difficulty-not to say impossibility-of making sure that no typhoid carriers and no persons suffering from a mild case of diphtheria or scarlet fever are ever employed in a dairy is self-evident. Outbreaks of diphtheria, paratyphoid fever, and other diseases have, in fact, been traced to certified milk. For this reason, as well as because of the relatively high cost of production, the use of certified milk remains limited.

Pasteurization. The process of destroying pathogenic bacteria in milk is by far the most satisfactory method of controlling milkborne infection. The use of a temperature high enough to kill most microorganisms but not so high as to produce radical alterations in the substance heated was first applied by Pasteur to preserving wines without destroying their original flavor or bouquet. Although still widely used in connection with bottled beer and wines, the process of pasteurization is now used chiefly for the treatment of milk.

The temperature to which milk is raised and the time for which it is held there are dictated by the heat resistance of the bacterium to be killed. From the beginning attention has been directed primarily toward the tubercle bacillus, and it has been found that this bacterium is killed by exposure to a temperature of 140° F. for 20 minutes. In practice, then, a temperature of 142° to 145° F. for a period of 30 minutes provides an adequate margin of safety, and these are the requirements that are usually specified. The technical aspects of such treatment of milk on a large scale are of prime importance, taking the form of prevention of foaming, proper design of valves to prevent "cool pockets" and dead ends, and the like. It might be supposed that a higher temperature or longer holding time resulting in a greater margin of safety would be desirable; this is not the case, for if either the time or the temperature is increased alterations take place in the physical state of the milk in which the fat is dispersed into smaller globules and will not rise to the top on standing.

Pasteurization may also be accomplished by the application of higher temperatures for shorter times, 160° to 161° F. for 15 seconds, in the so-called flash or HTST (high temperature short time) process. Unless the milk is preheated and the process precisely controlled, it may not be quite as effective as the holding process, and it has been reported that Brucella will survive HTST without preheating. There is also some tendency for the process to affect the physical qualities of the milk.

The plate count of milk is tremendously reduced by pasteurization, for not only are the pathogenic bacteria, such as the tubercle bacillus, *Br. abortus*, streptococci, and the like, destroyed, but the majority of other bacteria present as vegetative cells are killed also. There appears to be some question of the efficacy of pasteurization, particularly the HTST process, in the destruction of Q fever rickettsiae for, while this disease does not seem to be milkborne, rickettsiae introduced experimentally are not invariably destroyed by either pasteurization process.

The phosphatase test is based upon the presence of the heat-sensitive enzyme phosphatase in milk. Since 96 per cent of the enzyme is destroyed by heating to 143° F. for 30 minutes, the amount remaining in a pasteurized milk may be used as an indicator of the pasteurization as carried out. The enzyme liberates phenol from phosphoric-phenyl esters, and, as originally proposed, the test consisted of the addition of disodium phenol phosphate and Folin's reagent, incubating 18 to 24 hours, and reading the blue color developed.

Not all bacteria are killed by pasteurization. Besides the resistant spore-formers. aerobic and anaerobic, certain streptococci are able to survive the pasteurizing temperature. These are the lactic acid-formers rather than the pathogenic forms, and pasteurized milk sours in the ordinary way on standing, though a longer period is required i.e., its keeping qualities are improved. If higher temperatures are used (180° F.), the lactic acid bacteria are killed and proteolysis occurs as the milk spoils. Thermophilic bacteria are frequently present in pasteurized milk in great numbers, for the temperature of pasteurization is an incubation temperature for these microorganisms. The slight acid metallic taste occasionally noticeable in pasteurized milk is often attributable to their activity.

The presence of thermophilic and other thermoduric microorganisms in milk is taken as indicative of faulty farm sanitation, provided that the equipment of the processing plant is not contaminated with these forms as is sometimes the case. On the other hand, the presence of psychrophilic bacteria in pasteurized milk, such as Pseudomonas, Alcaligenes, and Achromobacter, not only detracts somewhat from the keeping qualities of the milk, but is almost always an indication of poor plant sanitation practices, i.e., represents post-pasteurization contamination.

The process of pasteurization, while effective in destroying pathogenic bacteria present in milk and, incidentally, increasing its keeping qualities, is not to be regarded as an excuse for the marketing of dirty and highly contaminated milk. There is some evidence, for example, that the growth of enormous numbers of bacteria, even though nonpathogens in the usual sense, is associated with summer diarrhea of infants. In most cases, therefore, sanitary regulations specify not only the allowable number of bacteria in pasteurized milk as delivered but also an upper limit for raw milk which is to be pasteurized.

The regulation of milk quality. The application of appropriate methods of rendering and keeping milk satisfactory from the hygienic point of view is, essentially, a social and legal problem rather than a scientific one. To this end appropriate ordinances are more and more generally incorporated into the legal structure of the community, and these, when adequately enforced, produce a marked reduction in milkborne disease. About half the cities of over 1000 population grade milk and permit the sale of one grade of raw milk and one grade of

pasteurized milk; of the total volume of market milk about 74 per cent is pasteurized, 99.4 per cent is from tuberculin-tested herds, and 35 per cent from abortion-tested herds. In general, sanitary regulations are somewhat more strict and more rigorously enforced in the large cities. In Chicago, for example, all milk is pasteurized, including certified milk, and this regulation is rigidly enforced.

Such practice has resulted in marked reductions in the number of bacteria in market milk. In 1901 the bacterial content of market milk in New York City varied from 300,000 in the coldest weather to 5,000,000 during the summer months; in Chicago (1904) the counts ranged from 10,000 to 74,000,000, and in Boston (1892) averaged 4,500,000. The incidence of milkborne disease has correspondingly decreased; for example, in 1907–1915 there were in Massachusetts 2215 cases of typhoid fever which were traced to milk, but in 1919–1923 only 297.

Milk products. Various foods made from milk, such as ice cream, butter, cheese, and the like, are potential vectors of disease when made from milk contaminated with pathogenic bacteria. The bacteria tend to die out upon storage, although it has been found that the typhoid bacillus will survive for three months or more in butter, and tubercle bacilli have been found in butter and certain quick-ripening varieties of cheese. Ice cream may serve as a vector for typhoid fever, streptococcal infection, etc. Pasteurization of the mix is customary and generally at higher temperatures than those used for milk. Human infections with foot-and-mouth disease have been traced to contaminated butter and cheese, but the public health significance of these findings is problematical.

Food Poisoning and Foodborne Infection 12, 13, 17, 42

The diseases transmitted by milk and milk products may be disseminated by a variety of other foods; in addition to these, however, other types of illness may result from the ingestion of contaminated foods which make up that group of affections designated as food poisoning. The kinds of

illness that may result from the ingestion of food may be summarized briefly:

- (1) Individual idiosyncrasies
- (2) Toxemia from foods, such as
 - (a) Foods naturally poisonous
 - (b) Foods into which poisons have been accidentally introduced

- (c) Foods containing poisons of bacterial origin formed by
 - 1. Clostridium botulinum
 - 2. Staphylococci
- (3) Foodborne infection, including both
 - (a) Bacterial infections, such as
 - 1. Typhoid fever, dysentery, and cholera
 - Salmonella infection
 - (b) Parasitic infections

In some instances food serves simply as a vector in the transmission of diseases such as the parasitic infections and the enteric diseases. In the remainder, however, the clinical manifestations are those associated with food poisoning proper vomiting, diarrhea, enteritis, and a greater or lesser degree of prostration. Although a number of types of food poisoning given above are not bacterial in origin, their clinical character may simulate bacterial food poisoning, and these must be considered in any attempt to ascertain the etiology of a given outbreak. Hypersensitivity to a given food substance, for example, is frequently manifested as vomiting, and an outbreak confined to a family may be the result of familial tendency; similarly, the gastrointestinal disturbances following the ingestion of naturally poisonous foods such as toadstools, or foods contaminated with poisons such as arsenic or cyanide, are often indistinquishable from those induced by some poisons of bacterial origin.

FOOD POISONS OF BACTERIAL ORIGIN

Poisoning with food containing toxic substances of bacterial origin is very common, probably more so than is generally recognized. The term "ptomaine poisoning" is a misnomer that is both misleading and inaccurate. The organic bases such as putrescine, cadaverine, methylamine, and the like, called ptomaines, which result from the bacterial decomposition of protein, are not toxic when given by mouth. Neither are other decomposition products toxic per os. While a partially decomposed food may be esthetically unattractive, the innocuous nature of the products of decomposition is obvious when one considers the advanced state of decomposition reached by some cheeses. Toxicity is, on the contrary, attributable to the presence of substances synthesized by the bacteria whose presence may or may not be associated with obvious evidence of decomposition of the food substance.

Botulism. Botulism is a highly fatal form of food poisoning resulting from the ingestion of preformed toxin of one or another of the types of Clostridium botulinum (Chap. Twenty-nine). These toxins are neurotoxins and among the most potent poisons known (Chap. Nine). The separation of these microorganisms into types, designated by capital letters, is made on the basis of the immunological specificity of their toxins; i.e., there is no cross-neutralization by antitoxin with the exception of the subtypes of type C. Botulism in this country is usually due to type A or type B; more recently type E, prevalent in Japan and also found in the Scandinavian countries, has been responsible for botulism in this country and Canada.

Cl. botulinum is commonly present in soil, existing as a saprophyte, for infections are extremely rare. Soil, then, represents the source of infection of food in the case of types A and B. Type E botulism has been associated with the consumption of fish products, and the microorganism has been isolated from the intestinal contents of fish from the Great Lakes, with an incidence as high as 9 per cent in Lake Michigan, and 57 per cent in Green Bay.⁴ A sixth type, type F, described in 1960 and associated with human botulism from liver paste in Denmark, has also been isolated from salmon from the Columbia River.¹¹

Given contamination of foods with Cl. botulinum, there are two prerequisites for the formation of toxin. First, since the microorganisms are obligate anaerobes, anaerobic conditions are required for growth, and these are provided in foods which are canned or sealed as in sausage casings, plastic wraps, etc. Second, a period of incubation is required, and this offers no difficulty for such processed foods are considered to be preserved and are stored, often for considerable periods of time, without refrigeration. Under such circumstances, the bacilli grow and form toxin, with perhaps only slight if any overt evidence of spoilage. As described elsewhere (Chap. Nine), the botulinum toxins are relatively resistant to enzymatic digestion, and in fact those of types A, B, and E may be split to lowermolecular-weight active fragments by proteolytic digestion and are, therefore, effective by the oral route.

Outbreaks of botulism occur as groups of small numbers of cases, the individuals who have consumed the toxic food. Botulism of types A and B in this country most often involves home canned foods, particularly neutral foods such as green beans which are difficult to sterilize and may have been put up by a cold pack method. Such outbreaks tend to occur in rural areas.

A typical history may include the serving of the food at fault, some agreement that it tasted a little abnormal, and discarding of the remainder, for example, by feeding it to chickens. Chickens are susceptible to botulism, a characteristic sign being paralysis of the neck muscles to produce "limberneck." Symptoms of human botulism follow within a day or two. Antitoxin is effective but only prophylactically, since symptoms are the result of nerve damage, which antitoxin cannot repair. If some of the food is available it may be injected into experimental animals, some of which are passively protected with antitoxin. Or the microorganism may be isolated and identified on the basis of its toxigenicity.

Until the appearance of type E botulism, commercially canned food had not been a cause of botulism for many years; regulations for commercial processing are laid down by the Food and Drug Administration and are highly effective. A total of 1561 cases of human botulism have been reported as occurring in this country between 1899 and 1963,35 including those due to type E since 1932. In Europe botulism has been more often due to sausage (hence the name) of one kind or another than to canned vegetables.

Type E botulism has involved fish and fish products for the most part, as indicated above. A raw fish preparation, izushi, has been the most common vehicle in Japan.⁴⁴ Uncooked fish has been involved in this country also, notably smoked whitefish (chub) from the Great Lakes, which had been packed in plastic bags and distributed through a supermarket chain.45 An outbreak due to canned tuna fish28 was the first instance of botulism from a commercially canned food in this country for 40 years. The spores of Cl. botulinum type E are less resistant to heat than those of the other types, and the bacilli grow in small pieces of muscle tissue at unusually low temperatures. 15

Staphylococcal food poisoning. Like botulism, staphylococcal food poisoning is a consequence of the ingestion of preformed toxin. The enterotoxin is usually produced by strains of staphylococci which are virulent by the usual criteria, i.e., golden-pigmented Staphylococcus aureus, strongly hemolytic on blood agar and coagulasepositive (Chap. Sixteen). In one series of such strains of hospital origin about 10 per cent were found to be enterotoxigenic.22 There seem to be no distinguishing morphological or physiological characteristics associated with enterotoxin-producing strains.

Production of enterotoxin is maximal on semisolid brain heart infusion agar⁵ and diffuses into the surrounding medium. Its action is central rather than local, and it produces vomiting and diarrhea in man after an incubation period of two to six hours, with recovery in 24 to 48 hours. The monkey is the only susceptible experimental animal. The enterotoxin is effective by the oral route in a dose perhaps ten-fold that required to elicit a response in man. A gastroenteritis is produced30 and the outward symptoms are much less marked than in man, usually consisting of vomiting a few times within a period of about five hours. It is also effective intravenously in the monkey. A kitten test has been described repeatedly, but it appears to be relatively nonspecific.

Enterotoxin has been prepared in a highly purified form and has been found to be a protein having a molecular weight of 35,000 to 40,000. It precipitates with specific antiserum⁶ in the gel diffusion test developed by Surgalla and his associates, and has been found to occur as two immunological types, designated enterotoxin A and enterotoxin B. More recently a third immunological type, enterotoxin C, has been described.³ The enterotoxins so differentiated do not appear to differ in their activity.

The foods involved in staphylococcal food poisoning are most often starch-containing materials such as custards used in cakes and eclairs, gravies, dressings, salads, etc., but enterotoxin is produced in meat also. The source of infection may be a product such as bulk eggs, but probably more often is an infected person, such as a nasal carrier or one having an open abscess, who prepares the food in question. Contamination may persist in inadequately

washed implements such as pastry-tubes. Custard fillings, salads, etc. are not cooked after preparation, and the penetration of heat into foods which are subsequently cooked is often insufficient to kill the relatively resistant staphylococci. Before it is consumed, the food may remain for some hours at room temperature, as on a shelf or in a showcase or en route to a picnic, to provide the incubation period necessary for growth and enterotoxin production by the staphylococci.

The identification of an outbreak of food poisoning as staphylococcal in etiology has usually been inferential: i.e., the vehicle is defined on epidemiological grounds and is found on culture to contain enormous numbers of staphylococci. Ordinarily, human volunteers or monkeys are not available to test the toxicity of the food or the enterotoxigenicity of the isolate. With refinement of the gel precipitation reaction as a slide double diffusion test, as little as 1 µg. per milliliter of enterotoxin may be detected and applied to appropriate concentrated extracts of the food in question.7 Under laboratory conditions, and using deliberately contaminated foods, enterotoxins A and B could be identified serologically in 40 to 70 per cent of cases. This kind of test has been described²¹ as more or less routinely applicable, but as yet has not been generally used.

This kind of food poisoning is very common, much more so in this country.²⁶ than in Europe, where foodborne Salmonella infections (see below) account for the great majority of food poisoning outbreaks.^{36, 38}

FOODBORNE BACTERIAL INFECTIONS

Whether other kinds of bacteria thought to be associated with food poisoning—because of their presence in very large numbers in foods incriminated on epidemiological grounds—produce enterotoxins is questionable. For the present such association, if it is truly etiological, may be considered to be foodborne infection

The foodborne infections are of two general kinds. On the one hand, a number of diseases, largely enteric diseases in which the oral route represents the usual

portal of entry, may be transmitted by food, but with the production of symptoms characteristic of those diseases. On the other, foodborne infection with some kinds of bacteria results in disease characterized by sudden onset, vomiting and diarrhea, and remission of symptoms within a relatively limited time—in short, a food poisoning syndrome.

The first kind of foodborne infection includes diseases such as bacillary and amebic dysentery, typhoid and paratyphoid infections, and cholera, in all of which the symptoms and course of the disease are characteristic of those produced by the microorganism and do not resemble those of food poisoning.

Foodborne typhoid, dysentery, and other diseases of the first group ordinarily occur on a small scale; the great epidemics are waterborne and, to a lesser extent, milkborne. Although limited in scope, foodborne infection plays an important part in maintaining some of these diseases in endemic form. The so-called residual typhoid, for example, which remains in spite of hygienic control of water and milk supplies is, in large part, foodborne. The employment of cooks and other food handlers who are typhoid carriers provides the opportunity for food infection and consequent transmission of the disease.

In contrast, a number of kinds of bacteria producing a foodborne infection with symptoms characteristic of food poisoning have been described, including streptococci and coliforms. Among such bacteria, an etiological relationship has been firmly established only in the case of Salmonella and Clostridium welchii.

Salmonella food poisoning. Of the Salmonella infections, one of three species is generally involved: Sal. typhimurium (Sal. aertrycke) and its varieties (newport, stanley, etc.), Sal. enteritidis, and Sal. choleraesuis (including Voldagsen and paratyphi C). Sal. typhimurium is the most frequently observed, while Sal. cholerae-suis is only rarely present. The ingestion of food containing large numbers of these organisms frequently results in the typical symptoms of food poisoning. No enterotoxic substance has been shown to be formed by these bacteria, and it is probable that actual infection takes place as indicated by the somewhat longer incubation period and the finding of the bacteria in the feces. The case fatality is variable, ranging from zero to 10 per cent. Almost any kind of food may serve to carry these microorganisms, although meats and other protein foods predominate.

Both Sal. typhimurium and Sal. enteritidis are commonly carried by rats and mice, and it is probable that in many cases these rodents are responsible for the infection of food. In others the source of infection may be a human carrier, and in many cases of meat poisoning it has been found that the meat was from a diseased animal.

As in the case of staphylococcal food poisoning, there is usually a history which includes a possible, or probable, source of contamination and storage of the contaminated food under circumstances allowing bacterial growth. The identification of the etiologic agent found in the food is a routine laboratory procedure, involving isolation and identification by the conventional cultural and serological methods (Chap. Twentyone).

Clostridium welchii food poisoning.25 The association of the sporulating obligate anaerobe Clostridium welchii, a causative agent of gaseous gangrene (Chap. Twentynine), was found by McClung³¹ in 1945 to produce food poisoning in man. The etiology of this kind of food poisoning is now well established, and outbreaks are reported with increasing frequency,37 probably a consequence of more careful search for this microorganism. In California the number of outbreaks of food poisoning attributable to Cl. welchii increased four-fold between 1960 and 1961, and in the latter year accounted for 17.5 per cent of all persons affected by food poisoning.29 Similar increases have been observed in England, with perhaps one-quarter of all cases of food poisoning being attributable to Cl. welchii.

Not all strains are active, and there seem to be no differential characteristics of food poisoning strains.²⁰ This kind of food poisoning has been produced experimentally in human volunteers, and it was found that cell-free culture filtrates or boiled cultures were not effective, indicating that this kind of food poisoning is a mild infection.¹⁴ Meat, particularly poultry, is most commonly the vehicle of infection. The food involved in

the first reported outbreak was chicken salad. Consistent with this, it has been found^{32, 41} that contamination with *Cl.* welchii is not uncommon in poultry.

Shellfish and disease. Shellfish, including ovsters, clams, and mussels, have been responsible for the transmission of enteric disease, especially typhoid fever and more recently infectious hepatitis, through pollution of areas in which they are grown or stored. They are commonly eaten in an uncooked or partially cooked condition which facilitates transmission of disease; it has been found that, while scalloped ovsters, fried clams, and clams in chowder are practically sterilized, steamed clams, fried ovsters, ovsters in stew, and mussels cooked in the usual ways are not freed from coliform bacteria. The shellfish, in the course of breathing and feeding, filter large quantities of water and readily take up enteric pathogens from polluted beds or when placed in brackish waters near sewage outfalls to "fatten," in effect concentrating the pathogen. They are similarly contaminated with viruses, as indicated epidemiologically by the occurrence of foodborne epidemics of infectious hepatitis.9 Various enteroviruses, including ECHO and Coxsackie viruses, have been isolated from ovsters taken from contaminated waters33 and. under experimental conditions, have been found²⁴ to take up poliovirus and Coxsackie virus, with persistence of the virus for as long as 28 days.

Imbibed bacteria, however, if not ejected very soon, are passed through the gastrointestinal tract and discharged in about five hours. The rapidity with which bacteria are passed through shellfish suggests the possibility that they may be cleansed of infection by storage in clean or even chlorinated water. The possibility is not only feasible but practiced, four days sufficing to eliminate practically all coliform bacteria. Essentially the same methods used for the bacteriological examination of water are applied to shellfish. The United States Public Health Service has suggested that not more than 50 per cent of the 1 ml. samples of pooled shell liquor and finely chopped tissue of 10 or more oysters, clams, or mussels should show coliform bacteria in the presumptive test, but the figure is a guide rather than a standard and is flexible.

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FOODBORNE PARASITIC **INFECTIONS**

These include the various flukes, tape worms, echinococcus, and roundworms that infect man. In these diseases the infective stage of the parasite is often present in a food substance as a consequence of its life cycle and the mechanisms involved may be found elsewhere (Chap. Thirty-four).

CONTROL OF FOODBORNE DISEASE

Foods constitute an unusually effective inanimate vehicle of disease, largely because pathogenic bacteria are able to multiply in them to produce toxins and/or give rise to large numbers as an infecting dose. Control of foodborne disease is concerned with the entire sequence of events from the harvesting of the food to its final consumption. In general, it consists of the prevention of contamination, and the destruction of possible contaminants by processing and/or inhibition of their growth in the interval elapsing between such contamination and consumption.

In some instances it is not possible to prevent initial contamination, as by trichina in pork or Cl. botulinum in fish, but for the most part contamination is of human origin. Such sources of contamination are infected persons who handle food. These persons may be casual or chronic carriers or may suffer from transient overt infections. It is possible to control such contamination in part by periodic bacteriological examination and their exclusion from occupations involving food handling. This is a function of health departments, but it cannot be invariably effective. Thus, while many state health departments maintain records of all chronic typhoid carriers and prevent their contact with foods by not allowing them to work in restaurants or other establishments concerned with food preparation, periodic examinations of food handlers may well miss transient infections or carrier states.

Foods may be examined for sanitary quality in terms of the kinds and numbers of microorganisms present. This not only allows an estimate of their quality,39 but also permits inferences as to their history.

It is possible, then, to set up microbiological standards for foods, as is done for water and milk, which may be put upon a legal basis and be enforceable by agencies at one or another level of government, such as the Food and Drug Administration. The introduction of new methods of food processing, as in the case of frozen foods, raises new questions of processing and standards. 18, 19

While the foregoing approaches to the control of the microbiological quality of foods can be applied collectively, essential elements of such control are necessarily individual matters. The exercise of appropriate care in the domestic preparation of food-its storage under refrigeration where indicated, etc. - must be done at the individual and family level.

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- 334
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Chapter Thirteen

IMMUNITY

Antigens, Antibodies, and the Antigen-Antibody Reaction^{3, 5, 18, 38, 62}

It has been common knowledge for many years that recovery from certain of the infectious diseases is accompanied by the development of an enhanced resistance, and second attacks of a disease once overcome are not common. This enhanced resistance. or immunity, is specific—i.e., an individual immune to one disease may be no more than ordinarily resistant to others—and is variable from one disease to another, some giving rise to a "solid" immunity of long duration while in others the immunity is imperfect or partial and transient. The specific immune state supplements the complex of factors which make up nonspecific resistance to infection, and even a solid immunity may be broken down by fatigue, malnutrition, and similar factors which are not consistent with a state of physiological well-being.

Specific immunity is a consequence of the reaction of the host to the microorganism and/or its products and provides the basis for prophylaxis of disease or infection. Its development during the course of disease is the primary determining factor in the outcome of the infection.

Historically, immunity, or immunology, was concerned primarily with disease, but it became clear that the basic principles have general biological application that has as vet been limited. Immunological specificity appears to be intimately related to the basis of biological individuality, and makes possible a precise and sensitive characterization of the larger molecular constituents of protoplasm. As such, immunological methods become unique analytical methods applicable to problems such as protein synthesis, blood and other tissue incompatibilities, and phylogenetic relationships, as well as to certain kinds of noninfectious disease or manifestations of disease such as the hypersensitivities.

Here the subject material of immunology will be considered under the general heads of antigens, antibodies, the antigen-antibody reactions, and effective immunity to disease. The differentiation is somewhat artificial and analogous to the separation of virulence and resistance. Antigenicity is demonstrable only by the immune response, and, conversely, antibody is formed in response to the antigenic stimulus.

Antigens 21

An antigen is customarily defined as a substance which, when introduced into the tissues of an animal, provokes an immune response, demonstrable after a latent period by the appearance of antibody in the blood and other body fluids which reacts specifi-

cally with the antigen, by a specific immunity to an infectious disease when the antigen is a pathogenic microorganism and/or its products, or by an increased reactivity, or hypersensitivity, to the antigen.

This definition is that of a so-called com-

336 ANTIGENS

plete antigen which both stimulates antibody production and reacts with the antibody so formed. Other substances have a more limited antigenicity in that, while they react specifically with antibody, they are unable to stimulate antibody formation. These substances are partial antigens or haptenes, and when they are found in microorganisms after destruction or removal of complete antigenic substances, they are called residue antigens.

The partial antigens, or haptenes, are, in turn, subdivided into two groups by some workers. One of these is made up of the haptenes which react with antibody in vitro to give the usual serological reactions (Chap. Fourteen), and the other includes those which react with antibody, but without overt evidence of the reaction, and the antigenantibody reaction is demonstrable only indirectly as an interference or inhibition phenomenon. This last, as will appear, is a consequence of the structure of the haptene and has no relation to its antigenic specificity.

Relative antigenicity. The property of complete antigenicity is variable in that some substances are "good" antigens and give a marked immune response, while others are "poor" antigens and stimulate only a low-grade immune response characterized by, for instance, a relatively ineffective and transient immunity or small amounts of demonstrable antibody, etc. Substances such as ovalbumin, serum globulin, and diphtheria and tetanus toxoids are good antigens, but hemoglobin and nucleoproteins and their constituent histones are poor antigens.

Relative antigenicity must also be evaluated in terms of the animal species in which the immune response is provoked and the way in which the response is assayed; in fact, in some instances it may be difficult to demonstrate antigenicity, or lack of antigenicity, because of variation in the immune response of the test animal. In general, the rabbit and chicken are good producers of precipitating and agglutinating antibodies, but there are differences between the two with respect to the kind of antigen, e.g., the antigenicity of hemoglobin is more readily demonstrable in the chicken than in the rabbit. In contrast, the guinea pig and the rat are not good producers of these antibodies, but the guinea pig is readily sensitized to anaphylactic shock by extremely minute amounts of antigen, and the rat gives a good immune response assayed as protective antibody.

Similarly, certain of the high molecular weight polysaccharides, such as the pneumococcal polysaccharides, are antigenic in man and in the mouse, but not in the guinea pig or rabbit. Relative antigenicity, then, eludes precise definition.

Adjuvants.³² A good immune response requires exposure of the antibody-forming mechanisms to the antigen for an appreciable time, even though the actual synthesis of antibody may be very rapid, and adequate amounts of antigen are required. In the first instance, a single inoculation of an antigen that is rapidly dispersed in the tissues and eliminated elicits only a minimal immune response, and the same amount of antigen is much more effective when given in successive inoculations. In the second, the degree of the immune response is directly related in a limited way to the amount of antigen administered; e.g., the relation between the amount of diphtheria toxoid administered and the antitoxin titer produced is linear over a limited range.

When the antigen is particulate or in a relatively insoluble form it tends to remain in the tissues for longer periods and so provides a more prolonged antigenic stimulus. Killed bacteria are usually good antigens, and living attenuated microorganisms (Chap. Seven) often produce an actual infection in which the amount of antigen is increased over that inoculated and the antigen persists to give a prolonged stimulus. The antigenicity of soluble antigens, or even bacterial vaccines, may be enhanced by the use of adjuvants which precipitate or coprecipitate the antigen in a relatively insoluble form, or otherwise delay its absorption. An antigen depot is established, often at the site of inoculation, from which antigen is slowly dispersed to provide a prolonged antigenic stimulus. A variety of substances have been used as adjuvants, most commonly alum, calcium phosphate, and water-in-oil emulsion containing the antigen. The effectiveness of the last is increased by the inclusion of killed acid-fast bacilli such as tubercle bacilli or related saprophytic forms-the Freund adjuvant. Antigenicity is appreciably, or even markedly, enhanced by such adjuvants. For example, two inoculations of alum-precipitated diphtheria toxoid will give an immune response as good as that produced by three inoculations of the fluid toxoid, and the antigenicity of a poor antigen

may be demonstrable when an adjuvant is used.

While a prolonged antigenic stimulus is undoubtedly a major factor in the effect of adjuvants on antigenicity, other factors are involved also. There is, for instance, reason to believe that the immune response is enhanced by an irritant effect of the antigen or adjuvant to give an inflammatory reaction and accentuation of the response of the antibody-forming mechanisms. This is the rationale behind the so-called nonspecific protein therapy of disease in which substances such as typhoid vaccine are administered. Still other factors are involved also, and the nature of the enhancement of antigenicity by adjuvants is not fully understood.

PROPERTIES OF ANTIGENS

Antigenicity may be further characterized as the kinds of substances that are antigenic, *i.e.*, the properties associated with antigenicity. These are, in general, of three kinds. First, antigens are substances of natural origin that are of relatively large molecular size; second, they are digestible, at least to a certain extent, by enzymes present in the body tissues; and third, they are foreign, or contain structures foreign, to the antibody-producing mechanism of the test animal.

Size and nature. Complete antigens are usually naturally occurring proteins containing a full complement of amino acids, and certain high molecular weight polysaccharides of natural origin may also function as antigens. It is possible that high molecular weight polymers may be antigenic, but with the possible exception of polyvinyl pyrrolidone⁴⁹ none has been found to be antigenic. Large molecular size invariably accompanies the ability to stimulate antibody formation, though all large molecules are not antigenic, and it is probable that molecular weights of 10,000 or greater are essential to antigenicity. There is, in fact, some rough correlation between relative antigenicity and molecular weight in that antigens having molecular weights of less than about 40,000, such as lysozyme, protamines, histones, and insulin, are usually poor antigens. Consistent with this, the antigenicity of a protein disappears rapidly on enzymatic hydrolysis, and proteoses and polypeptides are not antigenic.

A number of derived proteins, notably gelatin, are not antigenic, or only feebly antigenic in conjugated form, even though of high molecular weight. Gelatin is deficient in aromatic amino acids, and it has been supposed that an adequate content of aromatic radicals may be associated with antigenicity, but there is little definitive evidence for this.

Conjugation of an antigenic protein with another substance usually does not impair its antigenicity, and may even enhance it, but often alters its specificity (see below). In fact, many naturally occurring antigens are complexes of protein with other substances, commonly polysaccharides, and good antigens, such as ovalbumin and globulin, often contain carbohydrate as an integral part of the molecule. There are a number of exceptions among naturally occurring complexes; the nucleoproteins are not better antigens than the histone moiety, and a number of glycoproteins are poor antigens.

Degradation. The susceptibility of the antigen molecule to enzymatic degradation in the tissues appears to be a highly significant element of antigenicity. Particulate antigens, such as microorganisms, are phagocytosed and broken down both physically and chemically, and soluble protein antigens are readily metabolized. Not only is it not necessary that the intact antigen molecule persist as such, but there is reason to believe that the immediate antigenic stimulus is, in fact, an active fragment of the antigen which affects directly the antibody-forming mechanism. Such active fragments have not, however, been detected in the products of enzymatic degradation of antigens in vitro. The effect of an antigen depot produced by the inclusion of an adjuvant with the antigen is not one of preventing degradation, but rather of reducing the rate at which degradation occurs to provide a continuing source of antigenic stimulus over a longer time and more efficient utilization of antigen.

The significance of antigen degradation is illustrated in another way in the case of pneumococcal polysaccharide. As indicated above, this substance is antigenic in the mouse, but it is metabolized only very slowly by the tissues and is excreted largely unchanged in the urine. As a consequence, an apparently anomalous situation occurs in which the polysaccharide stimulates antibody formation only when given in minute

338 ANTIGENS

amounts. When larger amounts are administered, the antigen accumulates in the tissues where it is directly demonstrable, blocking the "detoxifying" mechanisms. The animal becomes refractory and will no longer form antibody, a state that has been called "immunological paralysis" by Felton.

Alien nature. The third essential prerequisite of antigenicity is that the antigen be foreign and distinguishable as "not-self" by the antibody-forming mechanism. 12 As pointed out elsewhere (Chap. Nine), the distinction between self and not-self is not made by embryonic or fetal tissue, and the property of making the distinction is usually not fully established until some time post partum, so that the animal immature in this respect is unable to form antibody. This does not invariably hold true, for the fetal tissue of some animal species, notably the fetal lamb, 68 has been found to be fully competent to form antibody. The animal mature in this respect reacts to the foreign nature of the antigenic substance by the formation of antibody except in the extremely rare condition of hypogammaglobulinemia.

The foreign quality of antigens may be of varying degree, ranging from that of antigenic substances of phylogenetic origin widely different from the recipient animal, to isophile antigens or iso-antigens occurring among individuals of the same species and within single individuals. The alien nature of antigens is reflected in the specificity of the antigen-antibody reaction, and widely different antigens appear to be quite unrelated immunologically, closely related antigens show cross-reaction by varying degree with appropriate heterologous antibody and behave as poor antigens when they are closely related to constituent antigens of the recipient animal.

ANTIGENIC SPECIFICITY

In general, antigenic substances from one species of animal stimulate antibody formation in another animal species, and antigens of plant origin are antigenic in animals. The relation between antigen and antibody is highly specific, as indicated above, in that antibody produced in response to the antigenic stimulus reacts specifically with the homologous antigen, and the antigen reacts

specifically with homologous antibody. In a sense the serological methods (Chap. Fourteen) used to demonstrate the antigenantibody reaction are analytical methods, sensitive, roughly quantitative, and highly specific for components of protoplasm which are antigenic.

Species specificity. Serum proteins are among the most commonly used antigens and may be used to illustrate specificity. Serum proteins from the horse, sheep, chicken, man, etc., are antigenic in the rabbit, while rabbit serum protein is not. Similarly, rabbit, horse, etc., serum protein is antigenic in the chicken, and the homologous chicken serum protein is not. Specificity is also expressed in the reaction of antigen with antibody. An antiserum to, for instance, horse serum protein prepared in the rabbit will react in vitro only with homologous horse serum protein antigen and will not react with serum protein from the chicken, man, cattle, etc. This kind of antigenic specificity is commonly referred to as species specificity although it obviously extends beyond the limits of species, through genera, and kingdoms, if plant antigens are considered also.

On the other hand, specificity is less sharp between antigens from closely related sources in that cross-reactions occur; *i.e.*, antiserum to chicken serum protein will also react with pigeon serum protein; antiserum to chicken egg albumin will react with duck egg albumin; antiserum to sheep serum globulin with beef serum globulin, etc.; but the heterologous reaction is weaker than that to the homologous antigen in that it occurs to lower titer than with the homologous antigen. Similar immunological relations occur among plant proteins as, for example, among seed proteins.

Phylogeny. It is a matter of no small interest that the immunological relationships among living organisms parallel their phylogenetic relationships based on a comparative anatomy.³⁴ Among the primates, for example, it was shown by Nuttall at the turn of the century that the Simiidae (gorilla, chimpanzee, orangutan) are very closely related to man in the antigenic specificity of blood proteins; the old world monkeys (Cercopithecidae) are not quite so closely related; the Hapalidae (marmosets) are still more distantly related; and the lemurs, which are Lemuroidae rather

than Anthropoidae, are unrelated to man. Similar relationships occur among other phylogenetic groups, plants as well as animals. It would appear to, and does, follow that antigenic specificity is genetically determined, and this is readily demonstrable within fertility groups; e.g., races of experimental forms such as Drosophila. Paramecium, fowl and other birds may be differentiated on this basis with segregation of antigenic specificities, and the immunological method has become a powerful tool in genetic studies as a measure of individuality. Genetically determined antigenic specificity is of practical significance in man in connection with blood groups and the incompatibilities observed in tissue grafts (see isophile antigens below).

Antigenic specificity has practical application for forensic purposes in the identification of blood protein in blood stains in criminal inquiries and in other areas such as the detection of adulteration of beef with horse meat, etc., and is fully acceptable as legal evidence. For such purposes it is essential that the antiserum used for the identification of unknown antigen be highly specific and without significant cross-reaction. The identity of the antigen may be suggested by relative titers with respect to the homologous antigen, but the specificity of the reaction may be established by absorption of antibody reacting with the heterologous antigen (see below) in some circumstances, or by the choice of animal immunized. For example, it is easy to establish that a blood stain is fowl blood rather than human blood, but the antiserum to human blood will react with the blood of some monkeys as indicated above. Crossreactions do not occur if the antiserum is prepared in the animal whose blood antigens show cross-reaction; thus, while antiserum to sheep serum globulin prepared in the rabbit will react also with beef serum globulin, if the antiserum is prepared in cattle there will be no cross-reaction.

Isophile antigens. Antigenic specificity, then, is at least a part of the basis of biological individuality, and its significance in this respect is underscored by the occurrence of antigenic differences between individuals of the same species alluded to above, the isophile antigens, *i.e.*, those which occur within the species, but not in all individuals of the species. The most important of these are

those characterizing the blood groups and those concerned in the reaction to tissue grafts.

Blood groups. 37, 61, 92, 93 It was found by Landsteiner in 1901 that incompatibilities observed in human blood transfusions have an immunological basis, and that human blood occurs in four immunological groups representing the possible combinations of two erythrocyte antigens. In the international system of nomenclature the antigens are designated A and B, and the combinations A, B, AB, and O, the last lacking both antigens. Antibodies corresponding to these antigens occur in the serum of individuals lacking the homologous antigen; i.e., the antigen and corresponding antibody do not co-exist in the same blood. The serum of the individual having type O blood contains both antibodies, ab; that of the individual having type A erythrocyte antigen, the antibody b; etc. The A antigen is further subdivided into two antigens, A_1 and A_2 ; the type A blood, then, may be A_1A_1 , A_2A_2 , A_1A_2 , A_1O , or A_2O , and so on, in combinations with the B antigens in type AB.

Many additional erythrocyte antigens have been described also. Two designated M and N occur separately and in combination with one another, and with the A and B antigens to give 12 antigenic types of erythrocyte, neglecting the subdivision of A. Another group of erythrocyte antigens, the Rh antigens, so-called because the first of the series was found by Landsteiner and Wiener in the rhesus monkey, also occurs. Antigens of this group are related to erythroblastosis fetalis, a hemolytic disease of newborn infants due to immunization of the mother, who lacks the antigen, by antigen occurring in the erythrocytes of the child by paternal inheritance, and destruction of the infant's red cells by the antibody. The original Rh antigen is designated Rh₀; rh' and rh" are two additional antigens; and Rh₁ and Rh₂ are complexes containing Rh₀ and rh' or rh" respectively. Still additional antigens are associated with this group; viz., hr' is the antigen found in cells lacking the rh' antigen, etc.

Still other blood group antigens have been described to give nine systems including the three noted above, AB, MN, and Rh, and it is probable that still other systems will be defined to include antigens which are not fitted into these groups.

Isophile erythrocyte antigens and the occurrence of blood groups are by no means confined to man and have been described in various lower animals, including rats, cattle, rabbits, and fish.^{77, 81}

There seems to be little doubt that the antigenic specificities of the blood group antigens are genetically determined, probably on a one-to-one relation between gene and antigen, and appear to combine in a simple Mendelian way. So the frequency of blood groups can be regarded as the phenotypic expression of human genotypes and has had valuable application in studies in human genetics. It has been found that there is appreciable segregation of such gene frequencies in subgroups or races of man. For example, 83 per cent of Eskimos have type M antigen in contrast with 57 per cent for Arabs, 28 per cent for English, and 2 per cent for Australian aborigines, and the frequency of type O blood varies from 85 per cent in American Indians, through 47 per cent for English, to 32 per cent for Russians. This subject is treated in detail by Boyd.4,6

Another application of the patterns of inheritance of the human blood group antigens is in forensic medicine in connection with disputed paternity. Comparison of the blood group antigens of mother, child, and possible father can show that a given individual could not have been the father, but cannot establish paternity.

The homograft reaction.^{2, 8, 66, 82, 85} When tissue is transplanted from one part of the body of a mammal to another, providing that it has not been injured in the process, the transplant, or graft, becomes vascularized, grows, and establishes itself permanently. Such a tissue transplant is an autograft; i.e., the donor and recipient are the same individual.

When a similar transplant is made from one individual to another of the same species, the grafted tissue also becomes vascularized, but after a few days or a week the graft tissue is invaded by lymphocytes and plasma cells, the blood vessels become dilated and engorged, the graft begins to be destroyed, and after two or three weeks destruction is complete and the graft is sloughed off. This kind of a graft is a homograft, and its rejection in the kind of reaction just described is the homograft reaction. If a second attempt is made to implant a homograft from the same donor, the reaction is

accelerated, and the graft rejected even more rapidly.

A third kind of tissue graft is the heterograft in which the donor and recipient are of different species. Reaction to the heterograft is even more violent than to the homograft, and the graft is rejected.

The homograft reaction is the consequence of an immunological response to the presence of isophile antigens in the donor tissue, but not present in, and therefore foreign to, the recipient. This effective immune response appears to be largely a hypersensitivity of the delayed type (Chap. Fifteen). Conversely, the graft tissue responds immunologically against the tissues of the recipient, but it is not clear to what extent this affects the rejection.⁷⁶

The isophile antigens concerned are, like the blood group antigens, genetically determined. This was shown in 1927 by the work of Loeb and Wright, who found that homografts between individuals of strains of guinea pigs so highly inbred as to be practically identical genetically took as well, or almost as well, as autografts. When tissue transplants were made between individuals of different inbred strains, they behaved as homografts. Further, the F, generation hybrids of such inbred strains accepted tissue grafts from either parent, but transplants from a hybrid to either parent behaved as homografts, indicating that neither parent contained relevant antigens foreign to the hybrid, but the hybrid offspring contained relevant antigens foreign to each parent which were contributed by the other parent. Human individuals are too heterogeneous genetically to behave in this way, but homografts between identical twins behave as if they were autografts.

The question of tissue incompatibility has been studied at some length in the mouse, and the genetic determinants of the relevant antigenicities are known as histocompatibility genes. A number of these have been described and designated H-1, H-2, etc.; of these H-2 has been the most intensively studied.⁷³

Prior inoculation with a suspension of living tissue homogenate accentuates the homograft reaction just as does previous tissue transplant, but it has been found that prior inoculation with a brei of killed tissue gives a variable, but sometimes enhancing, effect favoring the growth of a tumor homograft and possibly also of normal tissue

homografts. The mechanism of this enhancing effect is obscure. Some workers believe that the killed tissue induces a humoral (circulating antibody) rather than a cellular (Chap. Fifteen) immune response which favors survival of the graft by walling it off, while the living tissue gives a primarily cellular immunity that results in acceleration of destruction of the graft tissue. In this view, the humoral response, if it can be this sharply differentiated, would serve to isolate the graft from the antibody-forming mechanism in the same way that organ-specific and other iso-antigens are isolated.

Iso-antigens. In addition to isophile antigens discontinuously distributed among individuals of the same species, the individual also contains antigenic substances to which he will give an immunological response. These are iso-antigens.

The apparent contradiction is resolved by a more precise definition of the alien nature that is a prerequisite to antigenicity by further specifying, as indicated earlier, that the antigen is foreign to the antibodyproducing mechanism of the animal. Thus substances associated with reticular tissues or highly specialized cells and organs may be effectively isolated from the antibodyforming mechanism and retain sufficient alien nature to function as iso-antigens. There are a number of examples of such iso-antigens. It has long been known, for instance, that thyroglobulin is antigenic within the species, that lactating animals may be able to produce antibody to their own caseins, and that viper serum will protect against the venom of the snake as effectively as the best immune serums. Further, certain organs and tissues contain antigens, which may or may not be organ- or tissuespecific (see below) but which function as iso-antigens.

Auto-immune disease. 41, 47, 80, 84 Under circumstances which are not as yet well understood, an individual may form antibody against his own tissues, resulting in auto-immune disease or collagen disease. The latter term indicates the common involvement of the connective tissue, and disease may take a number of forms, including hemolytic anemia, disseminated lupus erythematosus, idiopathic Addison's disease, Hashimoto's disease (chronic thyroiditis), and possibly also rheumatoid arthritis. The antibodies so produced are demon-

strable in a variety of serological reactions (Chap. Fourteen) such as gel precipitation, complement-fixation, and fluorescent antibody staining. A multiplicity of antibodies is usually found in auto-immune disease, including anticytoplasmic and antinuclear antibodies, and antibodies to leucocytes, red cells, and platelets. Of such antibodies, those to various nuclear components have been of considerable interest, and there is evidence that a delayed hypersensitivity reaction may be involved also.⁶⁵

Heterogenetic antigens. While antigenic substances are, as described above, usually sharply specific for the species or even for the individual, certain antigens occur in different, often widely separated, species, that have the same or closely related specificities. Of these antigens the α-and β-crystalline of the lens protein of the eye, the Forssman antigen, and the organ- or tissue-specific antigens are among the best known.

Forssman antigen. The most extensively studied of the heterogenetic antigens is the common, or closely related, antigenic specificity which is named after its discoverer, Forssman. It is sometimes known as heterophile antigen although it is only one of a group of these substances.

Forssman antigen is present in a variety of phylogenetically unrelated organisms including dogs, cats, sheep, horses, certain fish eggs, turtles, and toads, and certain bacteria including strains of enteric bacilli and pneumococcus, and some varieties of corn (maize). It is not found in rodents except the guinea pig and hamster, nor in rabbits, frogs, hogs, and most primates. When the antigen is present, it is usually found in tissues and organs rather than in the blood, and in the sheep is found in the erythrocyte but not in other cells. Application of the fluorescent antibody technique (Chap. Fourteen) has shown that the antigen occurs in the endothelium and adventitial connective tissues of blood vessels in organs, with some species differences such as its presence in the collecting tubules of the kidney of the guinea pig and chicken, but not in these tissues of the mouse, dog, and cat.87

For a time it was thought that the Forssman antigen was a lipid, since it could be extracted from tissues containing it in ethanol, but it now seems clear that its antigenic specificity is a function of a carbo-

342 ANTIGENS

hydrate group present in the antigen. Its specificity is related to that of the human blood group A substance, and both are related to a fraction of hog gastric mucin, probably through content of similar carbohydrate groups.

The apparently random distribution of Forssman antigenicity is of practical significance in the interpretation of certain immunological reactions. For instance, the hemolytic antibody that lyses the sheep erythrocyte is usually Forssman antibody; in fact, Forssman's original observation was the lysis of sheep red cells by antiserums to guinea pig tissue prepared in the rabbit.

antigens.20 Still Organ-specific antigens whose specificity crosses species lines are those associated with certain organs and tissues. Kidney tissue, for example, contains antigenicity common to this organ from different animal species as well as species-specific antigens. In at least some instances antigenicity is associated with the heavy protein fraction separable from tissue homogenates by high speed centrifugation. They may be in part mitochrondria or, in the case of virus-infected tissues, the viral agent, and from the latter point of view the normal tissue components represent artifacts.26,40

Immunological studies have shown that the heavy proteins from various organs have three kinds of specificity, viz., species specificity, organ specificity related to the same organ in other species, and specificity for the organ of the one species. By the use of labeled antibody as by iodination with I¹³¹ or diazotization with S³⁵-containing compounds, it is possible to show that antibodies to the tissue-specific antigens localize in those tissues, usually in the vascular bed, and tissue-specific lung, liver, and kidney antigens may be demonstrated in this way.⁶⁰

Antibodies to such organ- and tissuespecific antigens may have a cytotoxic effect on the tissue in the intact animal. The kidney has been the most thoroughly studied in this connection, and heterologous nephrotoxic antibody has been found to produce nephritis with gross changes in the kidney, impairment of function as indicated by proteinuria, and histopathology of the glomerular epithelium. Similarly, continued inoculation with vaccines, such as rabies vaccine, containing brain and cord substance may affect the central nervous system by stimulating the formation of antibody which reacts with nerve tissue, and it has been shown that acute disseminated encephalomyelitis may be produced experimentally by the inoculation of homologous brain tissue with adjuvants.⁵⁴

Acquired tolerance. 15, 27, 28, 45, 51, 71 It was pointed out earlier that embryonic tissue does not form antibody, apparently because it is unable to distinguish the foreign nature of an antigenic substance not normally present in that tissue. It is a matter of particular interest that antigenic substances, even though they may be foreign in the sense that they do not occur normally in the individual, may be accepted as self when present in the embryonic tissue even after the capacity to make the distinction between self and not-self is developed. This acceptance, and consequent failure to form antibody to that antigenic substance even though the antigenicity of other foreign substances is unimpaired, has been called an acquired tol-

The naturally occurring phenomenon was demonstrated in 1945 by Owen,81 who found that sibling cattle twins had the same blood types much more often than expected on the assumption of a random segregation of the relevant genes. This was found to be a consequence of a common, though not invariable, occurrence of a common blood supply to the twins, resulting in a natural graft of blood-forming tissue to produce a double blood type or blood type chimera. Having been implanted in the fetal organism, and thereafter continuously present, this tissue was not subsequently recognized as not-self, and an acquired tolerance, or lack of immune response to the otherwise foreign antigen, persisted after the capability of giving an immune response had developed.

The same phenomenon is demonstrable also in the response to tissue transplants, twin cattle siblings reacting to the homograft as if it were an autograft, presumably as a consequence of a "graft" in utero of epithelial tissue. The phenomenon is demonstrable experimentally by inoculating mice in utero with epithelial tissue and showing that adults of mice so treated respond to homografts as if they were autografts while untreated mice give the homograft reaction. Similarly, animals inoculated with antigens such as toxoids subsequently show a marked

impairment of ability to form antitoxin although the immune response to other antigens is not affected. The possibility of an immunological tolerance functional in persistent viral infections is a matter of some interest. 90

THE CHEMICAL BASIS OF SPECIFICITY^{19, 39, 42}

A large body of sound experimental evidence has established the fact that the specificity of antigens is determined by their chemical composition. Early experimental investigation of a wide variety of antigenic proteins has shown that immunologically identical proteins are, so far as can be determined, identical in composition; that antigenic proteins differing from one another in composition are also immunologically distinct; and that antigens showing some degree of cross-reaction are closely related in chemical structure. Conclusive evidence that immunological specificity is a property of certain atomic and molecular arrangements has been obtained through the study of altered specificity and artificial antigens.

Altered specificity. The specificity of an antigenic protein may be altered by heating, partial denaturation, treatment with formaldehyde, etc., in such a way that part of the original specificity is lost but species specificity remains although somewhat broadened. Heated rabbit serum, for instance, behaves as a foreign protein in the rabbit, and the antibodies produced will not react with unheated rabbit serum. Retention of species specificity is indicated by the fact that antibodies to the heated serum react with the homologous antigen and to only a slight degree with heated serums from other animals. Such heated proteins are known as coctoproteins. Treatment of a protein with iodine, nitric acid, or nitrous acid (the last to give the intermediate diazo compounds), on the other hand, alters the specificity of the antigen so profoundly that species specificity is largely destroyed. Nitrated rabbit serum, for example, acts as a foreign protein to the rabbit, and the antibodies developed will react not only with the homologous antigen but with nitrated protein derived from other animal species or even from plants. Since iodine, nitrate, and similar substances react with the protein by substitution in the benzene rings of the aromatic amino acids, these findings have been interpreted as indicating that species specificity is associated with aromatic radicals and their arrangement within the protein molecule, a conclusion which, as will appear, is no longer justified.

Largely through the work of Landsteiner and his colleagues in the preparation and study of a large series of artificial antigens, some aspects of the phenomenon of immunological specificity are now relatively well understood. It has been found that the species specificity of antigens may be altered in ways other than attacking the aromatic radicals. Acetylation with acetic anhydride, esterification with ethyl alcohol, and methylation with diazomethane, all changes in the salt-forming groups of the protein molecule, result in a change in specificity similar to that obtained by treatment with iodine or nitric acid. It is of particular interest that such a marked effect on specificity may be produced by the addition of the small acetyl, ethyl, or methyl groups to the large protein molecule. Even phosphorylation has been found to alter the immunological properties of egg albumin.

Artificial antigens. 42, 67 The influence of the addition of relatively simple organic radicals on the immunological specificity of antigenic proteins has been further elucidated through the study of the immunological behavior of a variety of compounds prepared from protein and the diazonium derivatives of a number of methyl, chlor, brom, and nitro substitution products of aniline and o-, m-, and p-aminobenzenesulfonic acid as well as the parent compounds, and others such as p-aminophenylarsenic acid, o-, m-, and p-aminocinnamic acid. From these and other experiments it is apparent that the immunological behavior of antigens is specifically modified by a relatively small part of the large protein molecule, and the specificity of the antigen is determined by the chemical structure of this part, cross-reactions occurring between antigens whose determinant portions are closely related aromatic groups. The spatial arrangement in the determinative groups, as well as their nature, is reflected in immunological behavior; the position, ortho, meta, or para, of substituted acid groups in the aromatic radical produces differences in specificity, the stereoisomers of tartaric

344 ANTIGENS

acid and p-aminobenzoylphenylacetic acid yield immunologically distinct antigens when coupled with protein, and the position of amino acids in peptide-azoproteins is a factor in determining immunological specificity. In the case of artificial antigens, a minimal number of determinant groups must be added to the protein moiety to alter its specificity, 10 to 20, the number depending upon the size of the molecule, but not all are functional in the combination with homologous antibody (see below).

If it be assumed that the amino acids comprising the peptide chains of the protein molecule are arranged in regular and recurring order and proportion and, consistent with present theory of protein structure, that these chains are folded, then it is apparent that the recurring combinations of amino acids in the folded chains will result in "nodes" of molecular structure and patterns of polar groups. It is supposed that this type of molecular structure confers immunological specificity on the protein molecule that is not combined with some other type of structure.

In any case, a relatively small portion of the surface of the antigen molecule serves to determine its immunological specificity. It might, therefore, be anticipated that a single molecular species of antigen might provoke the formation of more than one kind of antibody specificity, i.e., have more than one kind of determinant. There is reason to believe that this is true, at least to a limited extent. Among the naturally occurring antigens, the pneumococcal antigen includes both the polysaccharide haptenedetermined type of specificity and the species specificity of the protein moiety complexed with another carbohydrate. The former is quantitatively predominant, and early during the course of immunizing inoculations the antiserum is sharply type-

Figure 63. An example of a conjugated protein antigen prepared by coupling p-azophenyl arsenilic acid with a protein by diazotization.

specific, but on continued immunization antibody to the species-specific antigenicity becomes more and more evident. It is a general observation that hyperimmune serums are often not as sharply specific as antiserums obtained by a less prolonged course of immunization. Further, while the specificity of a protein antigen may be altered as just described, antibody may be produced to the original as well as the new specificity.

It may not be possible to distinguish multiple antigenic specificities in a presumably "pure" antigen. The differentiation of antigenic specificities by the procedures of antigenic analysis (see below) is dependent upon the independent occurrence of the component specificities. Any antigen regarded as immunologically homogeneous in that it has only a single demonstrable antigenic specificity may, in fact, have multiple specificities which occur only in this particular combination and are, therefore, not differentiable.

Haptenes. The immunological function of the haptenes; or partial or residue antigens, now becomes clear in their role as determinants of immunological specificity. The relative importance of haptene-determined specificity is very great, for a large portion of naturally occurring antigenic substances are conjugated antigens consisting of a protein moiety and a haptene, and the latter is often found in the form of residue antigen in microorganisms. The significance of haptene-determined specificity in infectious disease is illustrated in the pneumococcus, whose polysaccharide capsular substance provides the antigenic specificity required to produce an effective immunity to infection with these microorganisms.

Polysaccharides. 9 Of the residue antigens those of the pneumococcus have been the most intensively studied and for present purposes may be regarded as typical. These so-called specific soluble substances (SSS). which make up the capsule of the bacterium, have been found by Heidelberger, Avery, Goebel, and others to be polysaccharide in nature. Of the pneumococcal type-specific polysaccharides, that of type 3 has been the most thoroughly investigated and appears to be a polymer of aldobionic acid units (in this case glucose and glucuronic acid joined through a glucoside linkage, $4-\beta$ -glucuronosidoglucose, designated as cellobiuronic acid) having a molecular weight of 1000 to

5600. The nature of the other type-specific polysaccharides is not so clear. Type 2 polysaccharide hydrolyzes to glucose; type 1 is possibly a trisaccharide made up of two uronic acid (in part galacturonic acid) molecules, a third molecule containing nitrogen, and acetyl groups whose presence is of considerable immunological significance; and type 4 yields an amino sugar on hydrolysis. That such polysaccharide haptenes are not peculiar to the pneumococcus is indicated by the isolation of similar substances from a variety of microorganisms including yeasts and other fungi and certain metazoan parasites as well as many species of bacteria.

The presence of capsular polysaccharide material confers immunological specificity upon the pneumococcus types; the rough, nonencapsulated forms are species-specific, and no longer may be differentiated into types irrespective of the immunological type of the smooth, encapsulated parent form. The species specificity manifested by the rough forms, furthermore, appears to be intimately associated with the presence of yet another polysaccharide, the so-called C polysaccharide, which is common to the rough forms and which, like type 4 polysaccharide, hydrolyzes to an amino sugar but also contains phosphoric acid. The genetic transduction of pneumococcus types, then, would appear to be an alteration in the ability of the microorganism to synthesize one or another of the type-specific polysaccharides.

The immunological specificity of other bacteria, including types A, B, and C of Friedländer's bacillus, certain of the meningococcus types, staphylococci, streptococci, and other microorganisms, is similarly associated with the presence of polysaccharide haptenes. The nature of the linkage between polysaccharide and antigenic protein in the bacterial cell is not known.

It has been shown also that an effective antigen need not contain intact polysaccharide; Goebel has prepared an azoprotein antigen from horse serum and the glucoside of cellobiuronic acid whose antiserum will protect mice against type 3 pneumococcus infection. Similar protection is afforded against type 2 infection by an antiserum to an azoprotein antigen prepared from the glucoside of a *synthetic* isomeric aldobionic acid, gentio-biuronic acid (6- β -glucuronoside-glucose).

Lipids.91 Principally because of the ap-

parent solubility of Forssman antigen in organic solvents and the immunological activity of certain lipoidal preparations from tubercle bacilli, it has been thought that lipids might function either as complete antigens or as haptenes. The experimental evidence, e.g., immunization with mixtures of foreign serum and such substances as lecithin, cephalin, and cholesterol, has, however, been inconclusive, and it is therefore uncertain as yet whether or not lipids may determine the immunological specificity of antigenic proteins. A number of steroids have been found to act as haptenes when conjugated with bovine serum albumin to form a complete antigen.1

Drugs. An immune response, often manifested as a hypersensitivity, to low molecular weight substances, including antibacterial substances and other drugs as well as cutting oils and similar substances responsible for occupational dermatitis, occurs with some frequency. It is probable that the low molecular weight nonantigenic substance combines with protein of the recipient to function as a haptene and alter its specificity so that it becomes a foreign substance and antigenic to provoke an immune response. The antibody so formed reacts specifically with the drug or other substance functioning as a haptene, and allergic reactions occur. Such sensitization to penicillin, for example, is observed relatively often.

THE ANTIGENIC STRUCTURE OF MICROORGANISMS^{44, 50, 83}

The term antigen may be used loosely to mean any antigenic material or, more precisely, to mean a single antigenic substance which is immunologically pure in that it contains only a single antigenic specificity. The great majority of antigens occur in mixtures with other antigens, and microorganisms are such mixtures, containing more than one, often very many, component antigens. The microorganism, then, consists of a mosaic of independent antigens, each of which stimulates antibody formation and reacts specifically with its homologous antibody. Because of this, an antibody may be preferentially removed from an antiserum containing many kinds of antibodies by adsorption on its specific antigen to provide the basis for antigenic analysis (see below), and characterization of the microorganism by its content of differentiable antigens, *i.e.*, by its antigenic structure.

The distribution of antigens among microorganisms is analogous to that among higher forms, in that individuals, or more accurately clones, may be immunologically specific, and at the same time show cross-reactions with similar forms, but some microbial antigens are heterogenetic and occur in widely separated forms. With appropriate reservations, antigenic specificity may be taken as a measure of biological individuality among microorganisms just as in higher forms, and evidence continues to accumulate that it is genetically determined also.

Differentiation of antigens. Microbial antigens are separable into groups on the basis of chemical properties, especially resistance to heat (100° C.) denaturation, and on the basis of location in the cell structure. as well as by immunological specificity. Those associated with cell structure include the antigens present in bacterial flagella, the flagellar antigens, those present in the capsule or envelope, the capsular or envelope antigens, and those present on the cell surface proper such as certain of the typespecific antigens of the streptococci. None of these appear to be essential parts of the cell since they may be removed with no apparent effect on viability. The intracellular antigens of the cell substance proper are the so-called somatic antigens and, in some instances at least, appear to be integral parts of the cell wall. In the case of microorganisms containing endotoxins, the toxicity is intimately associated with antigenicity, and endotoxin and somatic antigen may be wholly or in part identical. In addition to these kinds of antigens, soluble antigenic substances secreted by the microbial cell, such as exotoxins, various toxic activities such as hemotoxins and cytotoxins, and enzyme or enzyme-like activities such as kinases, are used also in the immunological characterization of microorganisms.

Some of the variety of antigens that may be present have been alluded to earlier. The capsular antigens, which are usually polysaccharides, but may be polypeptides, have been described above in connection with haptene-determined specificity. The myosin-like flagellar protein, flagellin (Chap. Three), includes the heat- and alcohollabile H antigens, so-called because their presence in enteric forms is associated with motility and a spreading (Hauch) type of growth on agar mediums. The somatic antigens of the enteric bacilli are the lipidpolysaccharide-polypeptide complexes that are the endotoxins, or lipopolysaccharides (Chap. Nine), and they are called O antigens because they are present when the microorganism is nonmotile and growth on agar mediums is not spreading (ohne Hauch). The antigenic specificity of the somatic antigens is a function of the polysaccharide moiety.46,74 The exotoxins and other toxic activities have also been described elsewhere (Chap. Nine).

Characterization. The immunological characterization of a microorganism may be relatively simple, in that one or two antigens may suffice for differentiation, or complex and concerned with many antigens. The diphtheria bacillus is an example of simple practical differentiation and is identified by its production of immunologically specific diphtheria toxin, and other characteristics are peripheral to this one. The pneumococcus is somewhat more complex, for two kinds of antigens are involved, that of the cell substance proper and that of the typespecific capsular substance. Differentiation of streptococci makes use of four kinds of antigens, a polysaccharide group-specific antigen and at least three type-specific antigens of polysaccharide, protein, and nucleoprotein nature. The antigenic structure of the enteric bacilli is more complex. In the Salmonella group the antigens are separable into the flagellar or H antigens, and the somatic or O antigens, with the former subdivided into phase 1 and phase 2 flagellar antigens. The somatic antigens were arbitrarily numbered by Roman numerals (Arabic numbers are beginning to be used), and the flagellar antigens by Arabic numerals and by lower case letters. Using these symbols, the antigenic mosaic of a bacterium of this group may be given as an antigenic formula; the paratyphoid B bacillus, for instance, has the formula I, IV, V, XII: b - 1,2. The coliform bacilli are equally complex antigenically, containing in addition to H and O antigens, somatic surface or envelope antigens called K antigens, and the K antigens are subdivided into L, A, and B groups of antigens. The antigenic characterization of these and other microorganisms is considANTIBODIES 347

ered in subsequent chapters devoted to more detailed consideration of individual microorganisms.

Common antigens. The heterogenetic antigens of microorganisms are usually called common antigens, i.e., common to more than one kind of organism. The Forssman antigen is one of these and is found in some bacteria, but not others, as well as in higher forms as indicated earlier. Other common antigens are shared by microorganisms with other forms, such as the immunological relationship between type 14 pneumococcal polysaccharide and the human blood group A substance; and those of type 2 pneumococcus, type B Friedländer's bacillus, and other coliforms to certain species of yeasts and vegetable gums such as gum acacia. Other common antigens occur between microorganisms as widely separated as the cholera vibrio and Brucella and the plague bacillus and paratyphoid B bacillus. There are many such examples, and it is clear that certain antigenic specificities found in microorganisms are heterogenetic in the sense that they seem to occur at random.

Antigenic variation. The antigenic character of microorganisms is subject to variation (Chap. Seven), and this may be viewed as variability in the ability to synthesize antigenically specific substances. In bacterial variation of the S-R type followed immunologically, the microorganism may be successively "degraded" with the disappearance of antigens and the appearance of apparently "new" antigens. This has been taken by some as indicative of the occurrence of antigens in layers like the layers on an onion which may be peeled off in successive degradations to expose new antigens. With the exception of flagellar and capsular antigens, there is no substantiation for such a view. It is more probable that the effect is one of alteration in the ability of the microorganism to synthesize haptenes, and when it fails to produce a haptene, such as a carbohydrate, the antigenic specificity of the protein moiety appears as a new antigen, or a new antigen occurs as a consequence of synthesis of an altered haptene to give a different antigenic specificity to the conjugated antigen.

Antibodies78, 79

The immunological response of an animal to the initial injection of an antigenic substance is not immediate but, after a suitable time interval or incubation period, is manifested as an alteration in the properties of the blood serum with respect to the antigen. An immune serum, or antiserum, differs from normal serum in that it reacts, either in vivo or in vitro, with the homologous antigen. This property of immune serum is a consequence of the presence of antibodies, substances which are formed by the animal body in response to the presence of antigen in the tissues and which combine specifically with the antigen. Since antibody is found in largest amount and in most convenient form in the blood serum, the properties and nature of antibody may be considered in terms of serum antibody.

Nature of antibody. Antibody activity is intimately associated with serum protein, and antiserums may contain 1 to 5 mg. per milliliter or even more antibody protein. In general, the activity is localized in the globulin fraction of the serum protein, the first to be precipitated by sodium or ammonium

sulfate or ethanol in the cold, but usually the concentration range over which precipitation occurs is not sharp. When serum globulin is salted out, as by half-saturation with ammonium sulfate, all of the antibody activity is precipitated and occurs in both the euglobulin and pseudoglobulin fractions, and usually less than 50 per cent of the total globulin, even in a hyperimmune serum, is antibody of a given specificity.

On electrophoretic fractionation of serum protein, the globulin is separable into three fractions on the basis of mobility: α -globulin. β -globulin, and γ -globulin in order of decreasing mobility. Antibody activity occurs almost exclusively in the y-globulin fraction, but there are occasional exceptions in which the several antibodies produced in response to inoculation with a complex antigen may be segregated in different protein fractions. For example, in the guinea pig sensitized with tubercle bacilli, antibody to the tuberculopolysaccharide is contained in the y-globulin fraction, while that to tuberculoprotein is found in the α -globulin and even to a certain extent in the albumin fraction. 17

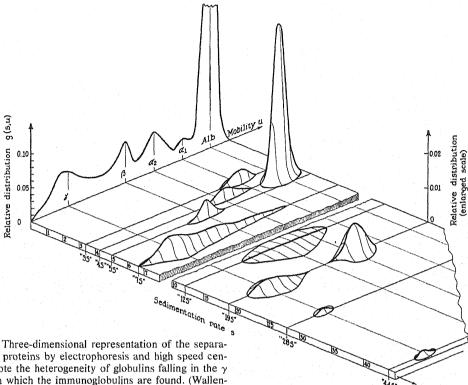


Figure 64. Three-dimensional representation of the separation of serum proteins by electrophoresis and high speed centrifugation. Note the heterogeneity of globulins falling in the y and β areas in which the immunoglobulins are found. (Wallenius: J. Biol. Chem.)

Or antibodies to the antigenic complex of the sheep erythrocyte which occur in the γ -globulin fraction may be found in separable parts of that fraction. Such variation in the distribution of antibody activity may be related to certain observed differences in the stability of antibody to different antigens. For instance, preservation of H-O antiserums with 0.5 per cent phenol is undesirable because on storage there is a preferential reduction and even eventual disappearance of antibody to the O antigen. Similarly, the antibody to O antigen is markedly reduced by heating to 70° C., while that to H antigen is not.52

In general it has not been possible to separate antibody activity from serum globulin, using a variety of purification procedures (see below). Thus, diphtheria antitoxin has been prepared as a pure protein crystallizing in thin plates and containing 700,000 to 1,000,000 antitoxic units per gram, and evidence such as this has led to the inevitable conclusion that antibody is modified serum globulin.

Immunoglobulins. 16, 23 Antibody activity is a property of more than one molecular species, which are separable by various

physical methods including electrophoresis, osmotic pressures, diffusion rates, viscosity, sedimentation by high speed centrifugation, and, more recently, immunoelectrophoresis (Chap. Fourteen), and are designated immunoglobulins. Two species have long been known, 7 S immunoglobulin having a molecular weight of about 150,000 or "classic" antibody; and 19 S immunoglobulin, or macroglobulin, having a molecular weight of about 1,000,000. The latter was originally described as formed by horses, pigs, and cattle in response to pneumococcus, though these animals form 7 S antibody to other antigens. It is now clear that 19 S antibody is not at all uncommon, and generally is formed early in the course of the immune response, while 7 S antibody is formed later.

The application of immunoelectrophoretic analysis to allow the separation of serum proteins of differing antigenic specificity and mobility has resulted in the differentiation of many serum proteins, including additional immunoglobulins. The terminology applied to the immunoglobulins has tended to become confusing, and an international nomenclature has been suggested under the auspices of the World Health Organization⁶³

which is gaining general acceptance. In this nomenclature, 7 S antibody, which has also had other designations, is termed IgG or γ G, the Ig or γ referring to immunoglobulin, and 19 S, or macroglobulin, is IgM or γ M. A third immunoglobulin, IgA, initially separated by immunoelectrophoresis and having a molecular weight of about 150,000, has been found in human and rabbit serum. A fourth, IgD, appears to occur in man and possibly also in the rabbit, but in very small amount and of doubtful antibody activity. The terminology of the three main classes of immunoglobulins is summarized in the accompanying table.

Properties of immunoglobulins. Analytical evidence indicates that the immunoglobulin molecule consists of four peptide chains, two heavy or long chains, and two light or short chains, joined by disulfide bonds. Certain biological properties suggest that the light chains may be double, and the molecule made up of six rather than four peptide chains, but the four-chain structure illustrated in the accompanying diagram is presently generally accepted as representing the basic structure of IgG. The heavy chains, also called A or H chains, have a molecular weight in IgG of about 50,000, are associated with the carbohydrate portion of the molecule, differ in amino acid content, and determine the distinctive properties of each class of immunoglobulin. The light, or B or L, chains have a molecular weight of about 20,000, occur in two electrophoretic mobilities, and are the subunits common to the three major classes of immunoglobulin.

The molecular weight of IgG agrees well with the sum of the molecular weights of its components. IgA has the same general structure, but the heavy chains have somewhat greater molecular weights, 60,000 to 100,000. IgM is considered to be a polymer of five basic four-chain structures in which the heavy chains have a molecular weight

of 65,000 to 70,000. Hydrodynamic data indicate that the IgG molecule is elongated, 250 to 300×40 Å, with an axial ratio of about 8; the IgM molecule is about the same diameter but longer. The prototype of the large antibody molecule is the Waldenström macroglobulin.

DEGRADATION. The structure of the immunoglobulins is inferred from study of degradation products of reduction with thiols, and digestion with papain or pepsin. Reduction splits sulfide bonds, and gentle reduction the interchain linkages, of which that linking the heavy chains is the most labile. Cleavage of the linkage between the heavy chains only gives two fragments, each made up of one light chain and one heavy chain, which retain antibody activity but behave as if they were monovalent; i.e., they will combine with antigen but lattices (see below) are not formed.

Enzymatic digestion splits the molecule across the heavy chains, leaving the disulfide bond between them intact. Cleavage by papain is at a point between the bond linking the heavy chains and those linking the heavy chains to the light chains. The molecule is, therefore, split into approximate thirds, giving two fragments containing a light chain and a portion of a heavy chain. and one fragment consisting of parts of two heavy chains and their associated carbohydrate linked by a disulfide bond, which is readily prepared in crystalline form. The light chain-heavy chain fragments are designated papain pieces I and II, and the other fragment papain piece III. Pieces I and II retain antibody activity but are monovalent, and piece III is immunologically inactive. These and other fragments of the immunoglobulin molecule are designated by the letter F: pieces I and II which have antibody activity are Fab, and the third fragment is Fc. Reduction of Fab with thiols splits the disulfide bond linking the light

Classes of Immunoglobulins

STANDARD (WHO	0)	OLD TERMINOLOGY	MOLECULAR WEIGHT	HEAVY CHAIN DESIGNATION
IgG or γG	**************************************	γ , 7 S γ , 6.6 S γ , γ_2 , γ_{ss}	150,000	γ
IgA or γA		$\beta_2 A, \gamma_1 A$	150,000	$oldsymbol{lpha}$
IgM or γM		$\gamma_1 M$, $\beta_2 M$, 19 Sy, γ -macroglobulin	1,000,000	μ

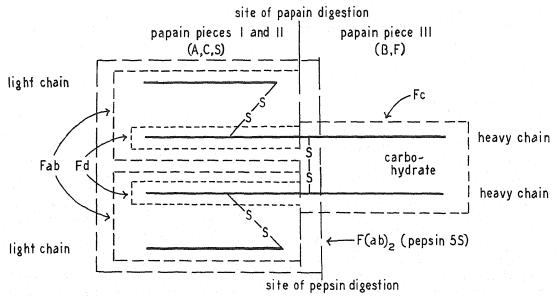


Figure 65. Diagrammatic representation of the presently accepted structure of the immunoglobulin (IgG) molecule. The antibody-combining sites are probably not adjacent as here indicated. (After Porter.)

chain and the portion of the heavy chain, with disappearance of antibody activity, and the heavy chain fragment is Fd.

Peptic digestion at pH 5 splits the immunoglobulin molecule on the other side of the disulfide bond linking the heavy chains to give a large fragment of about 100,000 molecular weight, the 5 S fragment, which has full antibody activity and, in the above notation, is F(ab')₂. The remainder of the molecule is accounted for as small fragments with no antibody activity. F(ab')₂ may be split by gentle thiol reduction of the disulfide bond linking the heavy chain portion of the complex to two components corresponding to Fab and retaining monovalent antibody activity.

Papain pieces I and II are separable by differences in electrophoretic mobility at neutrality into two types with respect to the light chain component. That of higher mobility is piece I from rabbit IgG and A in human and other serums, and that of lower mobility is piece II in the rabbit and C in the serum of other species. There are, then, two types of each class of immunoglobulin, designated K and L. As indicated above, the heavy chains are distinctive for each class. In the WHO nomenclature for molecular formulae, K and L light chains are κ and λ , and the heavy chains of the classes IgG, IgA, and IgM are γ , α , and μ respectively. On this basis molecular formulae can be written, that of IgG, for example, is $\gamma_2 \kappa_2$ or $\gamma_2 \lambda_2$ as the case may be; other designations and formulae are shown in the accompanying table.

BIOLOGICAL ACTIVITY. The biological activity of immunoglobulins is of two general kinds: their antigenic and their antibody activity. Regarding the former, the short chains are common to the known species of immunoglobulin, while the long chains are antigenically specific and differentiate the classes from one another. This is demonstrable by reacting papain fragments with specific antiserums; e.g., antiserum to light chain antigen will react with pieces I and II, but not with piece III, while antiserum to heavy chain antigen reacts with pieces I, II, and III and is class-specific, as well as giving cross-reactions between classes. The long chain antigens, then, con-

Immunoglobulin Formulae

PRESENT USAGE	ABBREVIATED NOTATION	MOLECULAR FORMULA	
7 Sy = type I	IgGK or yGK	$\gamma_2 \kappa_2$	
7 Sy = type II	IgGL or yGL	$\gamma_2\lambda_2$	
$\gamma_1 A = \text{type I}$	IgAK or yAK	$\alpha_2 \kappa_2$	
$\gamma_1 A = \text{type II}$	IgAL or vAL	α,λ,	
$y_1M = type I$	IgMK or yMK	$[\mu_2 \kappa_2]_5$	
$\gamma_1 M = \text{type II}$	IgML or yML	$[\mu_2\lambda_2]_5$	

tain both group and individual specific antigens. The contained carbohydrate does not appear to determine antigenic specificities. The heavy chain antigens are isophile antigens, and considerable interest has attached to their genetics.⁶⁴

All three classes of immunoglobulin have full antibody activity. As noted earlier, IgM is usually the first to appear during the course of immunization and is eventually replaced by IgG. The time course of IgA is not altogether clear, but it seems to follow IgM and may coexist with IgG. The classes differ in their functional antigenicity. In general, the early, but transient, IgM reacts well with particulate antigens such as bacteria, but poorly with soluble antigens such as toxins, while IgG is more effective as, for example, antitoxin, and appears to be concerned in the anamnestic reaction, i.e., in immunological memory, but IgM is not. It has been suggested that IgM represents a rapidly mobilized first line of immunological defense against bacterial infections. Antibody functional in hypersensitivities, e.g., reagin, appears to be wholly IgA.

IgG has been described as preferentially transmitted across the placental barrier, but immunoglobulin present in secretions such as tears, saliva, respiratory and intestinal mucus, and colostrum and milk, and known as secretory immunoglobulin, appears to be largely, if not entirely, IgA. Serum IgA, as described above, tends to be somewhat variable in sedimentation rate, possibly tending to form aggregates, but secretory IgA is homogeneous in this respect. Further, it contains an additional peptide chain, designated the T-chain, which appears not to be covalently linked with the remainder of the molecule. This "secretory piece" is considered by some to stabilize the antibody. The IgA present in secretions appears to be locally formed, at least in the case of that present in intestinal mucus. It is demonstrable in the antibody-forming cells of the lamina propria (Chap. Fifteen) by the use of anti-IgA antibody, often as fluorescent antibody staining (Chap. Fourteen), and has been shown to occur in the mucosal epithelial cells in the large bowel, suggesting a transepithelial pathway into the lumen of the bowel.24

It is apparent from consideration of the structure of the immunoglobulin molecule and the antibody activity of its fragments that (a) both light and heavy chains participate in the reaction with antibody and (b) that there are two antibody combining sites on IgG. Since IgM is a polymer of five such units, it might be supposed that it would have 10 combining sites, but this is not true for it is also divalent. The site of antibody activity at a point involving both light and heavy chains has been further substantiated by Singer through the development and application of the technique of affinity labeling. ⁶⁹

Antibody synthesis.^{75, 86} The outstanding differential characteristic of immune globulin is its property of reacting specifically with homologous antigen. Modification of normal serum globulin is such that this property is acquired, but the modification is of a sufficiently subtle nature that it is not detectable by the usual analytical methods.

It will be recalled that the basis of antigenic specificity is the chemical nature and configuration of a haptene or other small determinant portion of the antigen molecule. It may be taken to follow that the modification of serum globulin which gives it antibody activity can be inferred from the nature of antigenic specificity. The antigen-antibody complex formed when the two react with one another is held together by secondary valencies (see below) which are probably dependent upon the occurrence of one or more areas upon the surface of the antibody molecule in which the pattern of polar forces is a kind of mirror image of that determining antigenic specificity. There is no evidence that such an area is attached to the otherwise normal serum globulin. On the contrary, it seems to be built into the structure of the molecule, resulting, according to Pauling, from the way in which the peptide chain is folded, and the localized pattern of polar forces occurs in the same way as that determining the antigenic specificity of a pure, unconjugated protein. After folding has been completed, the configuration is stabilized by the insertion of -S-S- and hydrogen bonds.

Theories of antibody formation.^{7, 13} In a very real sense, in the stimulation of antibody formation, the presence of antigen acts as an inducer of the synthesis of immune globulin in a manner analogous to the function of the inducer of adaptive enzyme formation (Chap. Seven). The specificity of the product of such induced synthesis is

attributable to the template provided by the determinant portions of the antigen molecule. The most widely accepted theory of antibody formation was suggested by Breinl and Haurowitz and by Mudd at about the same time (1930-1931). According to this theory, antigen, and more recent evidence suggests an antigenic fragment, persists for extended periods in the antibody-forming cells (Chap. Fifteen) to provide the template against which specific antibody is formed.¹⁴ The striking analogy to the induction of adaptive enzyme formation has been extended in an attractively plausible theory of antibody formation by Burnet¹⁰ in which the initial antigenic stimulus functions as an inducer, and the enzymatic adaptation giving altered, or immune, globulin persists for an extended period after the inducer, or antigen, has disappeared.

The foregoing is known as the "instruction" kind of theory; i.e., the antigenic stimulus, whether it be a fragment of an antigen or derived from an antigen, is external to the genone of the antibody-forming cell. A second kind of theory, the "selection" theory, was proposed by Jerne³⁶ in 1955, and has been elaborated by Burnet and others. 11, 43 In this kind of theory the pre-existence of individual antibody-forming cells (Chap. Fifteen) which, in the aggregate, can synthesize all possible immune globulins, is assumed. The antigenic stimulus, then, results in the preferential proliferation of those having the immunological potentiality to react with the antigen. Because of the selective effect of the antigenic stimulus. this kind of theory has been called the "clonal selection" theory. It may be considered to be a sophisticated version of the classic side-chain theory of Ehrlich (see below). The obvious objection to it is the necessity for postulating the appropriate, and essential, random somatic mutations of mesenchymal cells, presumably during the course of embryonic development, to give clones of the required specific immunological potentialities.

A highly provocative theory has been advanced by Smithies⁷² based on analogy with viral replication. It is suggested that, since the synthesis of immune globulin is in all probability nucleic acid-directed, such altered nucleic acid may be transferred to potential antibody-forming cells, and that such immunological competence per-

sists through replication of such altered cells.

It is not yet possible to assay the validity of these various theories of antibody formation, but the generalities are not mutually exclusive and they may well be combined in time. The sites of antibody formation, the persistence of antigen in the cells and tissues, and similar factors are significant elements (see Chap. Fifteen).

Rate of formation. However this may be, the formation of antibody is, in large part if not entirely, a total synthesis as indicated by the extremely rapid incorporation of labeled amino acids from the amino acid pool, with a transit time of 40 minutes at the most, and possibly considerably less. during the stage of active antibody formation. The rate of formation of antibody is deduced from serum antibody titer after correcting for the catabolism of antibody already present whose half-life ranges from two to five days. It is usually more rapidly destroyed in smaller animals having higher metabolic rates, but exception is to be noted in the case of the human infant in which antibody persists as long or longer than in the adult in spite of a relatively higher rate of metabolism.

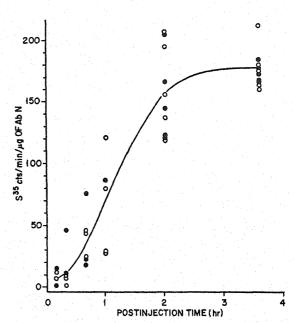


Figure 66. The rapid total synthesis of anti-bovine serum albumin antibody by the rabbit is shown by the rate of incorporation of S³⁵-labeled amino acids into the antibody molecule. (Taliaferro and Taliaferro.)

Antibody appears in the serum of the inoculated animal only after a latent period, rises rapidly and almost exponentially and falls off somewhat before reaching a peak. and then declines relatively slowly, the time dimension of the response varying with the antigen (Chap. Fifteen). This is the primary immune response. The latent period is regarded by some as the time required for the metabolism of the antigen, possibly to fragments which are the immediate inducers of antibody formation, and by others as a period during which a precursor is formed which subsequently reacts with antigen and is converted to antibody, and there is no doubt that a part of the latent period is required to saturate the tissues with antibody prior to the spilling over of the excess into the blood stream.

Secondary response. The secondary response is given by an animal having had a prior inoculation of the antigen, even months ago, and is characterized by a greatly reduced time required to reach peak serum titer - in the case of toxoids the time is reduced from two to four weeks to a few days—and the peak titer is often considerably higher than that reached following primary inoculation. It is obvious that the effects of the initial antigenic stimulus persist as a hyper-reactivity of the antibodyforming mechanism to the antigen, and it is for this reason that the secondary response is called the anamnestic, or remembering, reaction. The occurrence of the anamnestic reaction presents some difficulties for theories of antibody formation; in the adaptive

enzyme theory, for example, the adaptive enzymes catalyzing the synthesis of immune globulin are assumed to be self-duplicating under the second antigenic stimulus.

As indicated above, Pauling⁵⁵ has suggested that actual modification of the serum globulin occurs late in the process of synthesis, after synthesis of peptide chains is partially or largely complete. There is a certain amount of evidence for this in that it has been possible to denature, or unfold, normal globulin slowly by heating gradually to 57° C. and holding at this temperature for about two weeks in the presence of 2,4,6tri(p-azophenylarsonic acid) benzene as an "antigen," and cooling slowly to renature the globulin; the renatured globulin gave some antibody-like reactions but cannot be called antibody.56 The development of specificity as late as this in globulin synthesis is not consistent with the total, or practically total, synthesis of immune globulin from the amino acid pool. In this connection it may be pointed out that there is no evidence that normal globulin is a precursor of immune globulin, although labeled amino acids may be recycled from catabolized normal globulin and subsequently appear in immune globulin; nor is there any substantiation for the older idea that antigen is actually incorporated in the antibody molecule.

However the reactive sites are formed during the synthesis of immune globulin, there are only one or two, and probably not more than three, such areas on the surface of the molecule that are functional in that they will combine with antigen although

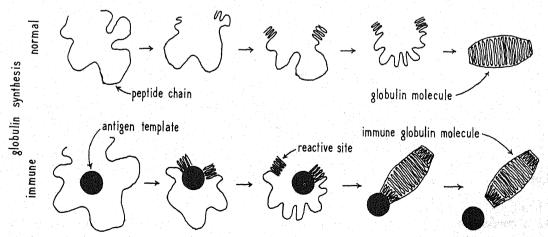


Figure 67. Diagrammatic representation of the sequence of events assumed to occur if antibody activity results from a directed folding of the peptide chain during synthesis. (After Pauling.)

354 ANTIBODIES

others may be present internally. It has been calculated that each combining site occupies a relatively small part, 1 per cent or less, of the total surface of the immune globulin molecule, and it is obvious that only a small portion of the molecule is directly concerned in the reaction with antigen. The number of such combining sites is spoken of as the valence of antibody, and, in general, antibody is monovalent or divalent in contrast with antigen which is multivalent (see below). When the antibody molecule is illustrated in diagrammatic form, the two reactive sites are usually shown at either end of the elongated molecule, but there is no reason to suppose that they occur in these positions or are even symmetrically placed.

Since the modification of globulin giving it antibody activity consists of the presence of "abnormal" localized patterns of polar forces on the surface of the molecule, it might be supposed that these areas may function as haptenes to alter the specificity of globulin as an antigen. If so, immune globulin might be antigenic in the animal producing it, and it should be possible to obtain antibody to antibody by immunization of other animals. This has not proved to be true, possibly because even if such reaction sites can function as haptenes, there are too few to alter the antigenic specificity of the globulin molecule.

Heterogeneity of antibody.^{22,89} At one time it was assumed that the several antibody activities demonstrable in the various serological reactions (Chap. Fourteen) are those of separate and distinct antibodies, that for example antitoxic and precipitating

antibodies to the same antigen are different, a supposition apparently supported by discrepancies in antibody titer measured in different ways. On the other hand, the essential similarities between certain of the serological reactions, especially precipitation and agglutination, are apparent and are reinforced by observations such as the passive agglutination of particles coated with soluble antigen by precipitating antiserum and the precipitating as well as antitoxic activity of antibody to exotoxins.

The common factor in the serological reactions, and in in vivo titrations of antibody activity, is the union of antigen and antibody (see below), but the consequences of that union are variable and determined by the way in which the test is carried out; e.g., is the antigen precipitated, is the animal protected against the toxin, etc.? Largely for physical reasons, some kinds of serological reactions require more antibody than others, thus leading to apparent differences in the amount of antibody determined. When it became generally clear that such discrepancies are, in fact, artifacts, the homogeneity of antibody to a single, immunologically pure antigen was formalized as the unitarian hypothesis. This simply states that a single antibody can produce various consequences following its union with antigen, and the different consequences observed are not evidence of the presence of more than one kind of antibody.

While this generalization holds true, it is equally clear that antibody is, in fact, heterogeneous, but in a different sense. Two kinds of heterogeneity are to be distinguished. The first is spurious in the sense

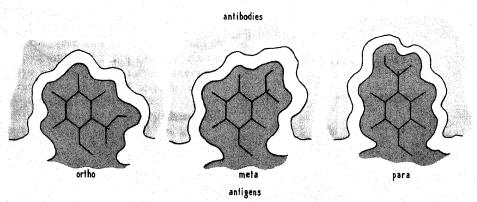


Figure 68. Diagrammatic representation of the effect of optical isomerism on antigenic specificity and the relation of the haptene to the site of antibody activity on the surface of the antibody molecule. (After Pauling.)

that the term antigen is often used collectively. While an immunizing preparation such as typhoid vaccine is called an antigen. it is in fact a mixture of the individual antigenic components of the mosaic making up the antigenic structure of the microorganism. Or the sheep erythrocyte, which contains Forssman antigen together with several isophile antigens, is also a mixture of antigens. When an animal is immunized with such material, the immune response is multiple, a group of differentiable antibodies, each specific for one antigenic component. are produced, and the antiserum is obviously heterogeneous with respect to its antibody content.

This may assume considerable practical importance. In the first example noted above, antibody to the H antigenic complex of the typhoid bacillus is not protective and is unrelated to immunity to the disease, while the antibody to the O antigenic complex is protective and the basis of an effective immunity. Since the titer of agglutinating antibody to the H complex is commonly much higher than that to the O complex. assay of the immune response by agglutination of the typhoid bacillus by the antiserum does not accurately measure immunity to the disease. In the second example, the titration of antibody that will sensitize the red cell antigen to lysis by complement (Chap. Fourteen) does not measure a single antibody, but rather the hemolytic antibody that happens to be present in highest titer.

Even when antibody is seemingly not heterogeneous in this respect, in that its formation is provoked by an immunologically "pure" antigen having but a single known specificity, more than one kind of antibody specificity, and therefore more than one kind of antibody, may be present in such an immune serum. It was noted earlier in connection with antigenic specificity that a single molecular species of antigen may have more than one demonstrable specificity and that what appears to be a single specificity may, in fact, be a complex antigen whose several specificities have not been found to occur independent of one another. Thus antibody to what is considered to be a single antigenic specificity may be heterogeneous, and the antiserum contains several antibodies specific for different parts of the antigen molecule. It is theoretically possible that such multiplicity may contribute to apparent differences in avidity of antibody (see below).

The second kind of heterogeneity is that of antibody to a single antigenic specificity. That some such variability occurs is already apparent from the earlier discussion of the properties of antibody. Antibody activity occurs in differentiable protein fractions such as IgM, IgA, and IgG, and antibody function becomes more efficient in time, with differences as much as 10,000-fold in combining power between early and late IgG; *i.e.*, there is a progressive change in the quality of the antibody.

Incomplete antibody.33 There is also a heterogeneity in antibody activity itself that may be divided into two kinds. The one is concerned with the number of reactive sites present on the immune globulin molecule. It was noted above that there may be one or two of these, and possibly rarely as many as three. Disregarding the last, antibody may, then, be monovalent or divalent (also see below). The former does not give the usual serological reactions and is demonstrable indirectly by its inhibiting or blocking effect on the reaction of antigen with divalent antibody and is also known as nonprecipitating, incomplete, or blocking antibody.

Truly monovalent antibody may be prepared by thiol reduction as indicated above. Unless the active fragments, Fab, are stabilized they will readily combine to form divalent antibody. Such combination may occur between Fab's of different specificity. For example, suppose two parent immunoglobulins having specificities AA and BB are reduced and allowed to combine. It is apparent that the combination occurs at random, since divalent molecules of specificities AA, BB, and AB are produced in the ratio of 1:1:2. Or immunoglobulin may be mixed with normal globulin, split, and allowed to combine to give monovalent antibody of the original molecular weight of the hybrid. There are some discrepancies between such monovalent antibody and naturally occurring blocking antibody, and it is not clear to what extent the naturally occurring blocking antibody can be considered to be monovalent in a strict sense.

Avidity. The other kind of heterogeneity in antibody activity is a consequence of how well or how poorly the reactive sites on the surface of the immune globulin mole-

cule are developed. The forces that bind antigen to antibody in the antigen-antibody complex are individually weak but in the aggregate provided a firm though dissociable union. When the reactive site of the antibody corresponds precisely to the determinant portion of the antigen, the maximum number of weak forces are operative, the complex is firmly bound, and the antibody is said to be highly avid. When the site is imperfectly developed, the complex is more readily dissociated, occasionally to such a degree that antigen and antibody may coexist in the same solution with only a small portion combined as the complex, and such antibody has a low avidity. Differences between IgM and IgG, and the increasing efficiency of the antibody activity of the latter, have been noted above. Antibody is, then, heterogeneous in this respect, and antibodies of varying degrees of avidity are found in the same antiserum.

Purification of antibody. The purification of antibody is a matter of its separation from other serum proteins, and the methods used are of two general kinds: the nonspecific chemical or physical methods and the specific methods which make use of homologous antigen.

The nonspecific methods commonly used are the precipitation of serum globulin by salting out, usually with ammonium sulfate, or by ethanol in the cold, and by electrophoretic methods. Serum globulin is precipitated at half-saturation with ammonium sulfate, achieved by the addition of an equal volume of the saturated solution to the antiserum, and adjusting to pH 7.8. The precipitate is washed, redissolved, and the ammonium sulfate removed by dialysis. Variations of this method are used commercially to effect a partial purification of antitoxins, and preparations of this kind may be given labels such as "immune globulin solution." Immune globulin is also precipitated by ethanol. A 1:1 dilution of the antiserum is chilled to 0° C. and 50 per cent ethanol added in small increments over a period of several hours until the final concentration is 20 per cent by volume. The temperature is lowered to -5° C., and after precipitation is complete, the precipitate is removed by centrifugation and dissolved in isotonic saline. Preparations of this general nature are often called "gamma globulin." The immunoglobulin species described above may also be separated from an immune serum for the study of biological, as well as physical, properties. Although they are differentiable by immunoelectrophoresis, column electrophoresis is not a satisfactory preparatory method, and IgM and IgA are not readily separated by centrifugation. The most commonly used procedure is fractionation by ion exchange chromatography. Nonspecific methods such as these separate the antibody-containing protein fraction of immune serum, but at best less than half of such preparations consist of antibody.

The specific methods of purification of antibody consist of reacting the antibody with homologous antigen to give an insoluble antigen-antibody complex which, washing, is dissociated to give free antibody. This approach is usually practical only when the antigen is, or can be made, insoluble so that it may be removed from the mixture after dissociation. Bacteria are, of course, particulate antigens and, after combination with antibody at neutrality, the complex is dissociated at pH 3, and the bacterial antigen removed by centrifugation to leave purified antibody in solution. Soluble antigens present a more difficult problem and a number of methods have been devised. Perhaps the simplest is the adsorption of antigen on ion-exchange cellulose. Antibody is specifically removed by combination with antigen on percolation through a column, and then preferentially eluted. Or the antigen may be thiolated, and the antibody separated from the antigen-antibody precipitate by removal of the thiolated antigen.⁷⁰

The Antigen-Antibody Reaction 48, 53, 88

The nature of the reaction between antigen and antibody has been of great interest since the phenomenon was originally described. The first comprehensive theoretical formulation was that of Ehrlich in 1906. He made the basic assumption that specific local reactive sites or receptors for food substances and also for antigens are pre-existing

on the body cells or cell structures. These receptors were regarded as having a relation to the cell structure analogous to that of side chains of complex molecules to the molecular nucleus, and this theory is known as Ehrlich's receptor theory or side-chain theory. According to this theory, when the receptors are saturated with antigen, particularly toxic antigen, the cell responds by synthesizing receptor substance in excess which then occurs free and is antibody. In order to account for the various manifestations of antigen-antibody union in the serological reactions, three kinds of receptors, of increasing complexity and designated as of the first, second and third orders, were postulated.

The combination between free receptors, or antibody, and antigen was considered by Ehrlich to be analogous to the neutralization of a strong acid by a strong base, and deviations from behavior predicted on this basis were accounted for by postulating varying degrees of avidity of antibody. This theory was slightly modified by Arrhenius in assuming that the antigen-antibody reaction is analogous to the neutralization of a weak acid by a weak base, so accounting for discrepancies such as dissociation of the antigen-antibody complex. These theories have little more than historical interest, but certain of the concepts and terminology, such as the term receptor, persist with modified meaning.

A more precise understanding of the nature of the antigen-antibody reaction began with Bordet's insistence in 1920 that it be considered as an adsorption phenomenon, and developed from the elucidation of the chemical basis of specificity described above and from the use of quantitative methods in the study of the reaction.

The antigen-antibody reaction may be differentiated into two stages, a primary stage concerned with the actual union, which occurs very rapidly, and a secondary stage which evolves more slowly as a consequence of that union. Here the primary stage will be considered, and the various manifestations of the secondary stage in the following chapter as the serological reactions. The secondary stage may be of such a nature that it is somewhat removed from the primary stage, but in the specific precipitation of antigen and antibody in the precipitin reaction, the precipitation is a direct exten-

sion of the primary reaction in that the antigen-antibody complex forms aggregates which appear as a visible precipitate. This reaction has been used almost exclusively in study of the antigen-antibody reaction, and for present purposes it is necessary to anticipate this serological reaction here.

Specificity. The nature of the specificity of the union between antigen and antibody and its surface, or adsorptive, character is indicated by the nature of antigenic, and by inference antibody, specificity as described above. It is dependent upon the juxtaposition and orientation of the haptenic or other determinant of specificity of the antigen with the reaction site on the immune globulin molecule. The antigenic determinant is visualized, at least in the case of artificial conjugated antigens, as projecting from the surface of the protein moiety, and it is believed by some that the corresponding reaction site on the homologous antibody may be in part a crypt in the surface of the globulin molecule; e.g., the shape of the haptene structure is important as well as its constituent atoms. The specificity of the union, then, involves orientation in at least two planes, and possibly also in three; and if the latter holds true, the specificity of the union is analogous to that operative in the formation of mixed crystals.

Intermolecular forces.⁵⁷ When the reaction sites of antigen and antibody have been brought into juxtaposition, they are specifically adsorbed to one another and held together by secondary valencies. These are not covalent bonds, but include electrostatic attraction between oppositely charged groups, dipolar interaction between polar but undissociated groups, hydrogen bonds, and van der Waals forces. None of these are strong bonds, and the last is the weakest, but when the stereochemical configurations of the bound molecule and receptor site are in juxtaposition, they are operative and form a firm, but dissociable, union. Of these, the coulomb forces between oppositely charged groups are possibly the most important as indicated by the marked effect of polar groups on specificity and the relatively high heat of the primary antigenantibody reaction.

Valence. The valence of antigen or antibody refers to a reaction site, the determinant or haptenic group of the former and the receptor site of the latter. It has been

implied earlier that antigen is multivalent in that a number of haptenic groups, such as arsenilic acid, must be attached to the protein moiety to alter its antigenic specificity. The number that are so attached to a pure protein of known molecular weight may be determined by analysis for, for instance, arsenic in the case of arsenilic acid conjugates. The number of such groups that are functional in the union with antibody may be shown by analysis of the antigen-antibody complex to be a function of the size of the antigen molecule. For example, the functional valence of ovalbumin having a molecular weight of about 40,000 is 5 or 6-i.e.this number of molecules of immune globulin may be attached to it when antigen is in excess-that of diphtheria toxin of molecular weight of about 70,000 is 8, and that of the high molecular weight (650,000) thyroglobulin is about 40. These are minimal values and represent the number of reaction sites that can unite with antibody simultaneously for antibody cannot react with sites too close together because of steric hindrance.

While complete antigens are multivalent. simple haptenes such as arsenilic acid are monovalent, and complex haptenes are multivalent. This is the basis for subdivision of haptenes into two groups as noted earlier. Monovalent haptenes will react with antibody, but complexes are not formed (see below), and the union is demonstrable indirectly by showing that the reacted antibody will no longer react with multivalent antigen. Haptenes may be made divalent by combining, for example, two arsenilic acid groups with some other compound, such as a dye, and such divalent haptenes give antigen-antibody aggregates. Most of the naturally occurring haptenes, such as polysaccharides, are multivalent and react with antibody to form aggregates even though they may not stimulate antibody formation.

It has been pointed out earlier that antibody may be monovalent or divalent, and the former is incomplete, inhibiting, or blocking antibody. In point of fact, there is no definitive evidence that literally monovalent antibody exists; more precisely, some kinds of antibody behave as if they were monovalent and inhibit or block reaction of antigen with divalent antibody. In the case of monovalent haptenes, valency is predetermined, but this is not true of incomplete antibody. It is possible, for example, that in some instances antibody may in fact be divalent but that the reactive sites are too close together to permit reaction with more than one molecule of antigen at the same time.

The existence of divalent antibody is, on the other hand, firmly established by extensive and precise studies showing that, in antigen excess, each antibody molecule combines with two molecules of antigen. The two antibody valencies are apparently always of the same specificity in a single molecule; *i.e.*, in the immune response to two or more antigens given simultaneously, a single antibody molecule does not have one valence specific for one antigen and the other specific for another.

Complex formation. The initial primary union of antigen and antibody occurs very rapidly and is usually complete within a few minutes, even at low temperatures. The aggregates which appear as a visible precipitate are formed over a longer period, as much as several hours. The aggregate material apparently consists of antibody molecules fairly tightly packed around antigen molecules, with some of the antibody molecules forming bridges between these units to give chains and a loose network of the complex. When either antigen or antibody is monovalent, or behaves as if it were, the available valences are satisfied, bridges, and therefore aggregates, cannot be formed, and there is no precipitate.

Composition of the complex. Whether or not visible aggregates of antigen and antibody occur, their relative content of antigen and antibody is determined by the proportions in which antigen and antibody are mixed. In this respect, three zones are to be distinguished, viz., the zone of antibody excess in which the proportion of antibody to antigen is large, and excess antibody is uncombined and demonstrable in the supernatant; the zone of optimal proportions, or equivalence zone, in which there is no uncombined antigen or antibody; and the zone of antigen excess in which antigen is present uncombined.

With respect to the formation of visible precipitate in the zones of antibody excess and antigen excess, two kinds of systems are differentiable. In one of these, the horse or H type, so-called because it is characteristic of the horse antitoxin-toxin system,

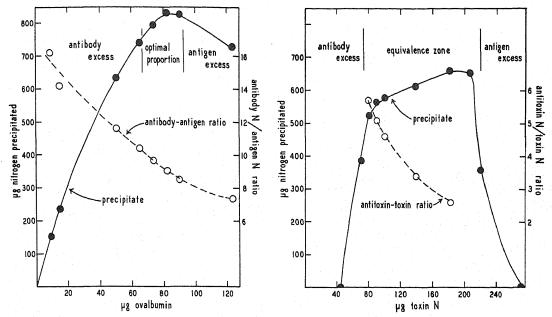


Figure 69. The two types of precipitin systems. Left, the "rabbit" type, in which the complex is not soluble in the zone of antibody excess; precipitate produced by adding varying amounts of ovalbumin to 1 ml. of rabbit anti-ovalbumin serum. (Data of Heidelberger and Kendall.) Right, the "horse" type, in which the complex is soluble in either antibody or antigen excess; total nitrogen precipitated from 1 ml. of diphtheria horse antitoxin by varying amounts of toxin. (Data of Pappenheimer and Robinson.)

precipitation is inhibited in an excess of either reagent. In the other, characteristic of most antigen-antibody systems and called the rabbit or R type because antiserums prepared in the rabbit are usually used, precipitation is not inhibited in the zone of antibody excess, and in this region all of the antigen is precipitated so that the amount of precipitate formed is determined by the amount of antigen added. These two types of precipitating systems are illustrated in the accompanying figure.

The failure to form precipitate, or other manifestation of the antigen-antibody reaction in the zone of antibody excess is called the prozone phenomenon. In the case of the toxin-antitoxin system, this is due to the formation of antigen-antibody complexes which do not aggregate. In practice, however, the prozone phenomenon occurs in all kinds of antigen-antibody reactions and even in in vivo protection tests (Schwellenwert). So it need not be due to the solubility of the complex in excess antibody. For example, there may be simply insufficient antigen present to give a precipitate. Or, when adequate amounts of antigen are present, as in bacterial agglutination, the particulate antigen may be rapidly saturated by antibody, leaving no functional valences available for the formation of antibody bridges; *i.e.*, the particles are soluble complexes. The inhibition of precipitation in antigen excess is theoretically due to the formation of soluble complexes, and this may be regarded simply as a consequence of the presence of antibody in amounts too small to aggregate the antigen present.

The bulk of the precipitate formed with soluble antigen is antibody. Measured by weight, the ratio of antibody to antigen may be quite small when the antigen molecule is very large, but with small antigen molecules it may be as much as 100, a consequence of surface-volume relationship in the antigen molecule. The molecular ratio of antibody to antigen may be determined in closely defined systems; with bovine serum albumin antigen and rabbit antibody, for example, the molecular ratio varies from about 6 in the zone of antibody excess to approximately 3 in the zone of antigen excess.

The optimal proportion or equivalence zone is, as indicated above, the antigenantibody ratio at which neither antigen nor antibody is present in uncombined form. In practice this is readily determined by the

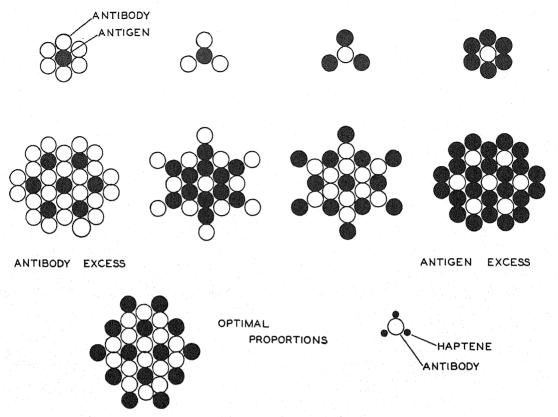


Figure 70. Diagrammatic representation of possible arrangements of antigen and antibody molecules in the complex. (Modified from Marrack.)

precipitin ring test (Chap. Fourteen) applied to the supernatant. This usually, though not invariably, coincides approximately with the antigen-antibody ratio giving a maximum amount of precipitate, and that at which precipitate first appears. The last criterion is the one usually used in toxinantitoxin flocculation. The titration may be carried out by using a constant amount of antibody mixed with varying amounts of antigen or by keeping the antigen constant and varying the antibody concentration. The former, designated the α -type procedure by British workers, is usually used, but the latter, the β -type procedure, is used for toxin-antitoxin titration. The two may give slightly different results, designated as the α -type optimum and the β -type optimum respectively, but usually a different β -type optimum is poorly defined or does not occur.

The ratio of antibody to antigen in the zone of optimal proportions is a constant for any given system; i.e., the ratio is the same when the concentrations are varied, and therefore constitutes one of the most

precise measures of antibody content of an antiserum. It occurs over a narrow range when a single antigenic specificity is concerned, and the antigen molecules are uniform, but takes the form of a plateau when more than one antigenic specificity is concerned, or when the antigen molecules, such as those of pneumococcal polysaccharide, vary in size and number of combining sites.

Mathematical analysis. A number of theories of the interaction of antigen and antibody have been proposed, and the modern theories date from Bordet's postulation of the reaction as an adsorption phenomenon. One of the first of these was that of Heidelberger,²⁹ who assumed that the reaction was similar to a chemical one between substances multivalent with respect to one another and behaved according to mass action to give a series of bimolecular reactions. Using the conventional symbols Ab = antibody and Ag = antigen:

 $Ag + Ab \rightarrow Ag.Ab$ $Ag.Ab + Ag \rightarrow AgAb.AG$ $AgAb + AgAb \rightarrow AgAb.AgAb$ and so on in the region of excess antibody, with the formation of visible aggregates having the general formula $(Ag_nAb_m)_x$. This leads to a general description of the precipitation reaction in the zone of excess antibody:

$$AbN = 2Rx - \frac{R^2}{AgN}x^2$$

where R = AbN/AgN and x = antigen precipitated. Since it is assumed that the reactions are irreversible, this holds only in the zone of antibody excess.

Hershey's^{30, 31} more intensive mathematical treatment is based upon the assumption the antigen-antibody union occurs as a series of reversible reactions which are unaffected by aggregation and may be represented as

On analysis, this approach gives the Langmuir absorption equation

$$R = \frac{ab[Ag]}{1 + b[Ag]}$$

in which R = the ratio of antigen in the precipitate [Ag], to the concentration of antibody in the supernatant, and a and b are constants. Equations of this type fit the linear relation between antigen-antibody ratios in the precipitate to the concentration of antibody in the region of antibody excess, and to the concentration of antigen present in the supernatant in the region of antigen excess.

Using a treatment identical with that of the dissociation of a polybasic acid, and assuming that antibody behaves as if it were monovalent, Teorell⁸⁸ arrived at the relationship

$$[Ab] = Ab - [Ag](p_1 + p_2 + ...)$$

when [Ab] and [Ag] represent the concentrations of free antibody and free antigen respectively in the supernatant, Ab the total amount of antibody present, and p_1 , p_2 . etc., represent the values $[Ab]/k_n$, $[Ab]/k_nk_{n-1}$, etc., in which k_n , k_{n-1} , etc., are the several equilibrium constants. This treatment allows a fairly accurate prediction of the course of the precipitation reaction over its entire range when reasonable as-

sumptions of solubilities and valence are made.

The most comprehensive treatment is that of Goldberg, 25 which is stochastic in taking into account the known heterogeneity of antibody present in immune serum by calculating the most probable distribution of molecular species by the methods used to study the distribution of molecular size of branched chain polymers. It assumes both divalent and monovalent antibody and multivalent antigen which react to produce branched chains until the system reaches a point at which it consists largely of relatively few large aggregates, and precipitation occurs, but no arbitrary parameters are introduced for the purpose of curve fitting. On this basis m_{ijk} , the number of aggregates composed of i divalent antibody molecules, j monovalent antibody molecules, and kmultivalent antigen molecules, is given as

$$\begin{split} m_{ijk} = & fG \; \frac{(fk-k)!}{(fk-2k+2-q-j)!k!q!j!} \; r^{k-l} \; p^{k+l+l} \times \\ p^{k+l+j-1} \; (1-p)^{+fk-k-l-j+1} \; (1-\rho \; pr)^{l-k+1} \; (1-\rho) \end{split}$$

when

$$i = k - 1 + q \text{ and}$$

$$O \le q + j \le fk - 2k + 2$$

and G = the number of molecules of antigen in the system, f = the valence of each antigen molecule, q = the number of free antibody valences in an aggregate, p = the proportion of antibody valences in the system that have reacted, ρ = the proportion of antibody valences on divalent antibody molecules, A = the number of divalent antibody molecules in the system, r = fG/2A, and s = fG/D where D = the number of monovalent antibody molecules.

Provided that the initial composition of the system, the valence of the antigen, and the extent of the reaction are known, this expression allows the calculation of the number of every kind of aggregate in the system, and the point at which very large aggregates are formed, and precipitation occurs.

The critical condition at which the complex takes the form of very large aggregates to give precipitation may be calculated from a simpler formulation derived from the above, viz:

$$\gamma p^2_c = \frac{1}{V-1}$$

when γ = the ratio of antigen valence to antibody valence, p_c = the critical fraction of antigen valences bound, and V = the valence of the antigen. It may be calculated that when the valence of antigen is 2, the formation of large aggregates occurs only in a mixture in which there is an equal number of antigen and antibody valences. With higher antigen valence, the range over which precipitation can occur becomes broad; for example, with an antigen valence of 6, precipitation can occur over a 25-fold range in the ratio of antigen valence to antibody valence, and with an antigen valence of 100, the range is 10,000-fold.

The occurrence of precipitation is also affected by the dissociation constant for, when the concentration of antibody is low relative to its dissociation constant, a significant portion of antigen and anti-body valences are uncombined. For example, with an antigen valence of 6, precipitation cannot occur when the antibody concentration is equal to or less than the dissociation constant. It follows also that the critical conditions for the formation of very large aggregates may occur in lower concentrations of antibody when the valence of antigen is large. This is consistent with the general observation that the larger the antigen, e.g., bacteria and erythrocytes, the less antibody is required and the higher the apparent antibody titer.

As pointed out earlier, the precipitin reaction may be regarded as a logical extension of the initial antigen-antibody union. It is perhaps incidental, as will appear, that toxin is neutralized by antitoxin, but the cytotoxic effects of antibody, or complement mediated by antibody, when the antigen is cellular would appear to be set somewhat apart from considerations such as these.

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Chapter Fourteen

IMMUNITY

The Serological Reactions

The variety of consequences of the primary union of antigen and antibody make up the so-called second stage of the antigenantibody reaction. These occur in vitro and, with the exception of certain kinds of protection tests (see below), are test tube reactions. Since the antibody is usually that contained in serum, these reactions are called serological reactions, and the general subject matter, serology. Strains of microorganisms differing in their antigenic structure and separated and identified by serological means are, for example, serological types or serotypes.

The serological reactions are extremely useful for a wide variety of purposes including serological identification of microorganisms and the resolution of their antigenic structure, measurement of the immune response, serodiagnosis of infectious disease by testing patients' serum for the presence of antibody to suspected etiological agents, etc.

The nature of the secondary stage of the antigen-antibody reaction is dependent upon the way the test is carried out, as noted earlier. In general, two kinds of effects are observed, the one an aggregation of soluble or particulate antigen in an overtly apparent way, and the other a cytotoxic action when the antigen is a living cell, that may result in death of the cell, changes in its permeability, or actual dissolution of the cell structure, all of which may be observed in vitro.

On the basis of the way in which the secondary stage occurs, several "kinds" of antibodies are distinguished. These are:

The precipitins or precipitating antibodies which aggregate soluble antigens.

The agglutinins which agglutinate or aggregate particulate antigens, including cells in suspension.

The antitoxins which specifically neutralize the pharmacological action of microbial toxins, especially exotoxins.

The *lysins* or antibodies which mediate dissolution of part or all of the structure of a cellular antigen by complement.

The opsonins which markedly facilitate the ingestion of cellular or other particulate antigens by the phagocytic cells of the body.

The protective or neutralizing antibodies which neutralize the infectivity of pathogenic microorganisms and protect the animal against infection and disease.

In addition to these, an animal may be made hyperreactive or hypersensitive to antigenic substances by a preliminary inoculation, and the consequences of antigen-antibody union are apparent only in living tissue, in vivo, or in vitro in certain kinds of preparations. This will be considered elsewhere (Chap. Fifteen), and the various serological reactions here. The specific technical detail of such reactions may be found in various compilations. ^{9, 27, 44}

PRECIPITINS

The precipitation of soluble antigen in the presence of homologous antibody by the formation of large aggregates of the antigen-antibody complex has been described earlier (Chap. Thirteen). In this kind of serological reaction the antibody functions as a precipitin, and the antigen which provokes its formation is known as a precipitinogen. The physical state of the antigen used for immunization is not of particular importance; antibacterial serums, for example, will give precipitates when mixed with preparations of the soluble cell substance of the microorganisms, and precipitins may almost always be demonstrated in lytic, antitoxic, opsonic and agglutinating antiserums.

Precipitation does not occur in the absence of electrolyte and does not require the presence of heat-labile substances such as complement.

The precipitin test is carried out with undiluted, or only slightly diluted, serum, but the antigen solution is diluted in series. The reagents may be mixed in the usual way, or the antiserum may be pipetted into very small test tubes and the solutions of diluted antigen carefully layered on top with no mixing. In the first instance a precipitate is formed which settles out like agglutinated bacteria, while in the second a precipitate forms at the interface between serum and antigen solution. The latter test is termed a "ring test." The end point of either test may be taken as the highest antigen dilution with which the serum forms an observable precipitate and the titer of the serum given in these terms. Potent antiserums may have extremely high titers, precipitating antigen in dilutions of 1:100,000 to 1:5,000,000.

quantitative precipitin reaction. 13 While such precipitin titers are indicative of the amount of antibody present, a more precise measure is the amount of antibody precipitated from a known amount of antiserum at the equivalence zone. The test is usually carried out by adding increasing amounts of antigen to a constant amount of antiserum. The end point is indicated approximately as that proportion of antigen and antibody which gives the maximum amount of precipitate, and precipitation usually appears first at this point. The equivalence zone, it will be recalled, is that proportion of antigen and antibody such that neither is in excess, i.e., present in the supernatant, and is demonstrated by application of the precipitin ring test to the supernatant. Precipitate formed at the equivalence zone may be removed by centrifugation and analyzed for nitrogen by the usual micromethods to give, by difference, the total antibody nitrogen present. In precise quantitative work the co-precipitation of other nitrogenous sub-

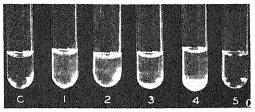


Figure 71. The precipitin reaction. Note the precipitate in tube 4 in maximal amount and the failure of precipitation to occur in the control tube (C) and tube 5, in which the antigen dilution is too great to form a visible antigen-antibody aggregate.

stances must be taken into consideration. The largest contribution of this kind is complement (see below), and for accurate results the antiserum examined must be decomplemented by treatment with some heterologus antigen-antibody system.

Precipitin reactions in gels.4,59,60 When antiserum is incorporated in a gel, such as gelatin or agar, and antibody allowed to diffuse into the gel, or when antigen and antibody diffuse toward one another in a gel, the precipitin reaction occurs as a band of precipitate. This phenomenon is known as immunodiffusion. The band is sharp when the proportion of reactants approximates that of the equivalence zone, but commonly this is not true, and the leading edge is sharp while the trailing edge is more diffuse. Since individual antigens form individual bands of precipitate with their homologous antibodies, the gel diffusion method allows the demonstration of antigenic heterogeneity because a mixture of antigens gives multiple bands of precipitation. The number of components so observed is a minimum, for there is no assurance that other components are not present but in too small amount to produce visible bands, and what appear to be single bands may sometimes be differentiated or resolved into more than one band.

There are a number of variations in the gel diffusion technique. The single diffusion method of Oudin makes use of antiserum-containing agar or gelatin in a small bore tube which is overlaid with a solution of antigen. On standing for some days, the antigen diffuses into the antibody-containing gel with the development of bands of precipitation. In a single antigen-antibody system only one band is formed, while multiple systems give a group of bands corresponding to the number of systems

present in appropriate amounts and proportions.

A double diffusion technique was developed by Ouchterlony in which antigen and antibody are placed in wells in an agar gel and allowed to diffuse toward one another to form a band of precipitate. Elek has used a similar method in which strips of filter paper soaked in antigen solution and in antiserum are placed on the surface of an agar gel. In the double diffusion technique multiple wells may be used; e.g., two antigen solutions may diffuse simultaneously toward the same antiserum. The bands of precipitate forming between each of the antigens and the antiserum fuse at their juncture when the two antigens are the same but cross independently when the two antigens are different.19 At the same time, multiple precipitates may be formed by a single antigen-antibody system, usually when the immunodiffusion is carried out with strongly unbalanced reactant ratios, or when the process is subjected to sudden changes in temperature.

Assay of antigen, or antibody, mixtures by precipitation in gels is based not only on the formation of successive bands of precipitate, but also on the rate at which they diffuse through the gel. The relation between the logarithm of the antigen concentration and square of the migration rate is linear under conditions of free diffusion, and superimposed bands may sometimes be resolved by adjusting the concentrations of the reactants. Oudin, for example, showed that the band of precipitate advances in the single diffusion method according to

$$h = k \sqrt[2]{t}$$

in which h = the distance traversed by the leading edge of the band, t = time, and k is a constant. Since k varies with the concentration of antigen and antibody, it is an index of the concentration of either reactant when the other is known. The viscosity of the gel, the presence of contaminants in the reactants, etc., affect k, and in general the quantitative approach to gel diffusion has not as yet given precise results.

The method has, however, been extremely useful qualitatively. For example, it has been possible to demonstrate by this means the presence of more than one antigenic component in highly purified diphtheria toxin otherwise apparently homogeneous. It also has practical application in this connection in the "in vitro virulence test" for toxigenic diphtheria bacilli; the bacteria are grown as a streak on an agar medium near to a strip of filter paper soaked in antitoxin, and a band of precipitate forms between the two when the bacterial strain is toxigenic.

Immunoelectrophoresis. 20, 31, 32 The technique of immunodiffusion may be combined with electrophoretic separation of one of the reactants, commonly the antigen. This is carried out by preparing the gel, commonly agar, in a thin strip on the surface of glass, applying the antigen in a well in the gel, and separating the components of differing mobility, the gel serving as a supporting medium. The separated material is then reacted with antiserum placed in a trench extending the length of the separation. Bands of precipitate occur about the separated antigenic components which may be stained, photographed, etc. This kind of immunoelectrophoretic analysis is usually carried out on a micro scale, i.e., in thin

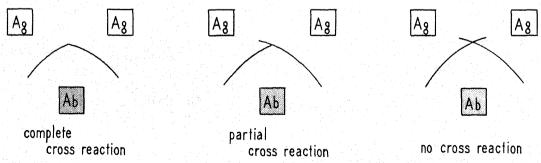


Figure 72. Diagrammatic representation of the Ouchterlony double diffusion technique with single bands of antigen-antibody precipitate. Left, the juncture of the bands indicates identity of the antigens; center, a partial cross-reaction; right, the independent behavior of the bands indicates no relation between the antigens. In all cases the antiserum contains antibody to both antigens.

367

layers of gel on a glass microscope slide. It has very considerable utility in the differentiation of differently charged antigens, as in ultrasonic lysates of bacteria, or the multiplicity of antigenic proteins present in serum.

AGGLUTININS

Precipitin reactions with haptenes. The precipitin reaction occurs not only between antibody and complete antigen but also with some partial antigens or haptenes. An antipneumococcus serum, for example. will react not only with a solution of the pneumococcus cell substance but also with pure capsular polysaccharide. When the haptene is of high molecular weight, as in the case of the polysaccharides, a visible precipitate is formed, but when it is a simple compound such as an organic acid or monosaccharide no observable precipitate appears. The reaction between antibody and such haptenes may, however, be shown by the fact that antiserums treated with haptene will no longer precipitate the complete antigen; the antibody has already reacted with the partial antigen.

Cross-reactions. The precipitin reaction, like the other immunological reactions, is highly specific, but cross-reactions are observed between similar antigens. With naturally occurring antigens, antigenic relationships tend to parallel phylogenetic relationships, as among the serum proteins of related animal species described elsewhere (Chap. Thirteen). Among artificial conjugated antigens, cross-reactions are associated with similarities in the structures of the attached haptenes. In precipitin and other immunological cross-reactions between naturally occurring antigens, it has not been possible to differentiate unequivocally between those occurring as a consequence of similar determinant structures and those that occur because of the presence of the same antigenic specificity in two otherwise differentiable antigenic mosaics.

In any case, the specificity of the precipitin reaction is such that it differentiates sharply between unrelated soluble antigens and is the serological reaction usually used for the identification of blood stains, etc., for forensic purposes.

AGGLUTININS

If the blood or serum of an animal previously immunized against a bacterium is mixed with a suspension of the microorganisms, the latter become immobilized and in a short time aggregate to form large clumps of cells. In the slide agglutination test, commonly used for rapid identification purposes, the antigen is used as a milky suspension and the antiserum undiluted or only slightly diluted: the suspended bacteria aggregate in the presence of homologous antibody within a minute or two to give a curdled appearance to the suspension. In the test tube these clumps settle out, and the turbid bacterial suspension is cleared with the formation of a precipitate-like mass of clumped cells in the bottom of the tube. This phenomenon is termed agglutination. and the bacterial cells are said to be agglutinated. The bacteria are not killed by agglutination and will, in fact, grow in immune serum although with altered morphology and the formation of long chains of bacillary forms. Living bacteria need not be used for the agglutination reaction, for dead bacteria are agglutinated as readily as the viable forms.

Although many species of bacteria may be clumped by "normal" serums in low (1:5 to 1:10) dilutions, the capacity of a serum to agglutinate bacteria is greatly enhanced by immunization; high-titered antiserums may be prepared which will bring about agglutination in dilutions of 1:20,000 to 1:50,000. The agglutination reaction is, then, an antigen-antibody reaction, and the antibody is designated an agglutinin. The antigen is sometimes termed an agglutinogen. The agglutinin does not require the cooperation of complement or other heat-labile substances, and inactivated serums will agglutinate to titer.

Not only bacteria but a variety of free cells, including erythrocytes and others, are agglutinated by normal and immune serums. The incompatibility of human blood groups is a consequence of the presence of hemagglutinins. Hemagglutinins are also formed by some bacteria and may possibly be instrumental in the formation of the thrombi observed in the blood vessels after death from certain of the infectious diseases.

Agglutination is highly specific, an antiserum agglutinating only the homologous antigen, and agglutination does not require complement. The reaction may be observed microscopically by mixing a suspension of bacteria and diluted antiserum on a slide but is most commonly carried out by mixing

the two in 0.5 to 1.0 ml. amounts in small test tubes and observing the formation of a precipitate. In the latter instance, varying dilutions of serum (frequently prepared in geometrical progression by mixing with an equal amount of physiological salt solution, i.e., 1:10, 1:20, 1:40, 1:80, etc.) are added to the bacterial suspension and incubated at 37° C. overnight or at 55° C. for two hours. The highest dilution showing observable flocculation is taken as the titer of the serum: a serum showing agglutination in a dilution of 1:10,000 but not in 1:20,000 is said to have an agglutinin titer of 1:10,000. Such titers are variable to some degree, depending upon the density of the bacterial suspension and other factors; light suspensions, for example, showing only a faint turbidity to the eye may give somewhat higher agglutinin titers than heavy suspensions.

Conglutination and conglutinating complement adsorption. 14, 38 It was observed by Bordet and Gay in 1906 that fresh serum of some animals will agglutinate erythrocytes treated with cow serum. The cow serum is the source of two factors: natural antibody for the erythrocytes, and the clumping activity, conglutinin. The reaction was termed conglutination to distinguish it from agglutination, which does not require complement. This reaction may be used as an indicator system in the same manner as the sheep-hemolysin system of the complement-fixation reaction, the presence or absence of free complement being indicated by conglutination or conglutination inhibition. Used in this way, it is called a conglutinating complement adsorption test (CCAT). It has become of renewed interest in connection with the serodiagnosis of Q fever and organisms of the psittacosislymphogranuloma group.

Passive agglutination.⁵⁶ Particles upon which soluble antigen is adsorbed acquire in a passive sense the antigenicity of that antigen and behave as if they were composed of that antigen. They agglutinate in antiserum to the soluble antigen, and the agglutination is said to be passive agglutination. This phenomenon illustrates the essential identity of precipitin and agglutinin, i.e., in the precipitin reaction molecules are aggregated or agglutinated, while in the agglutination reaction particulate material is agglutinated.

Antigen may be adsorbed on collodion, latex or similar particles of appropriate size, but red blood cells, usually of the sheep, have been the most commonly used for this purpose, and the reaction is a passive hemagglutination. Soluble antigens are usually adsorbed relatively rapidly, polysaccharides in 10 to 30 minutes, and proteins a little more slowly. The adsorption of protein may be facilitated by a preliminary treatment of the red cells with dilute (1:20,000) tannic acid. Stable preparations of formalinized, tannic acid-treated red cells may be used instead of fresh cells.21 A variety of antigens have been used, including polysaccharide haptenes, soluble antigens from microorganisms such as tuberculin, the O and Vi antigens of enteric bacilli, etc., bacterial toxins such as tetanus toxin, and certain viruses such as adenoviruses.

The original antigenicity of the red cell is not lost when a soluble antigen is adsorbed on its surface. This is a significant factor when sheep erythrocytes and rabbit antiserums are used, for these cells contain Forssman antigen, and antibody to this antigen is commonly found in serums of animals, such as rabbits, which do not contain the antigen. Consequently, sheep cells are often agglutinated in rabbit serum, and if these reagents are used, the antiserum must be treated with normal sheep cells to remove the Forssman antibody by absorption (see below) before use. Or this artifact may be avoided by using rabbit erythrocytes.

The passive hemagglutination test is often more sensitive than bacterial agglutination owing to the relatively large size of the antigen particle. Antibacterial serums having homologous antibacterial agglutinin titers of 1:5000 to 1:10,000 may agglutinate red cells sensitized with soluble bacterial antigen to titers of 1:50,000 or more.

The antiglobulin reaction. 15, 22 The antiglobulin reaction is a passive agglutination in which the antigenic cell or particle is treated, first with antiserum specific for its surface antigen, and then with antiserum to the serum globulin of the specific antibody. It is also known as the Coombs test. In the first stage of the reaction, an ordinary agglutination or passive agglutination, homologous antibody in the form of immune globulin reacts specifically with its antigen. When the immune globulin is thus specifically

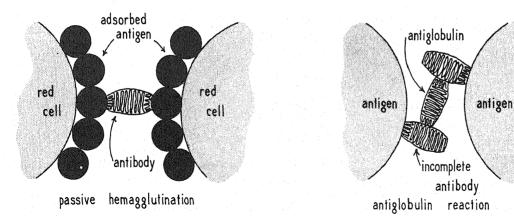


Figure 73. Diagrammatic representation of the passive hemagglutination reaction (*left*) and the antiglobulin reaction (*right*). Note that the antigen-antibody reaction is between antibody and adsorbed antigen in the first instance and between incomplete antibody globulin molecules and their homologous antibody in the second.

adsorbed onto the particle, the particle behaves antigenically as if it were serum globulin. On the addition of antibody to the globulin, antiglobulin, the immune globulin that acted as an antibody in the first instance now acts as an antigen, and the coated particles are agglutinated. While, then, in a technical sense the second reaction is specific, the specificity of the antiglobulin reaction as a serological reaction lies in the specificity of the original antigen and its antibody.

This reaction is particularly useful for the titration of incomplete antibody which will of itself not agglutinate a particulate antigen. It does, however, react with antigen to adsorb onto a particulate antigen, which then becomes susceptible to agglutination with antiglobulin serum. In other words, the incomplete antibody behaves as if it were monovalent, but as an antigen it is multivalent. For example, a bacterial antigen is agglutinated in its homologous antiserum by the complete antibody present. If, however, antibody to the immune globulin is added to the system, the bacterial cells which reacted with incomplete antibody will now agglutinate. Antiglobulin titers are frequently higher than agglutination titers, because the latter titrates only complete antibody, while the former titrates both complete and incomplete antibody. In some instances, notably that of Rh antibody, antibody is largely incomplete and agglutination occurs, for all practical purposes, in the antiglobulin reaction.

Cross-reactions. Although the agglu-

tination reaction is highly specific, certain cross-reactions between closely related bacterial species are frequently observed. Such reactions are attributable not to a lack of immunological specificity but to the immunological heterogeneity of the bacterial cell. As indicated earlier (Chap. Thirteen). the cell is made up of a variety of antigenic components, an "antigenic mosaic." Clearly, then, if the same component is present in each of two species of bacteria, an antiserum prepared against the one will agglutinate the other but generally to a reduced titer, i.e., only in the lower serum dilutions. This sharing of antigenic components and the resulting cross-reactions are particularly common among the gram-negative enteric bacilli. The agglutining responsible for such cross-reactions are sometimes referred to as "group agglutinins" and the phenomenon as "group agglutination."

Agglutinin adsorption. The direct demonstration of the antigenic heterogeneity of a bacterium and the consequent multiple antibody content of its homologous antiserum are made possible by agglutinin adsorption. If a heavy suspension of bacteria is prepared in diluted (commonly 1:5) antiserum, incubated for two to three hours and centrifugated, the supernatant diluted serum will be found to have lost its ability to agglutinate the bacterium with which it was absorbed; the agglutinins for that microorganism have been taken up by the bacterial cells, leaving other agglutinins intact. In practice it is necessary to absorb two or three times and, since the phenomenon is

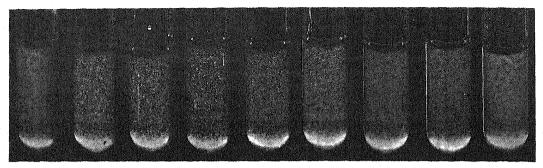


Figure 74. The macroscopic agglutination test—flagellar agglutination of Salmonella. The control tube (at left) contains bacterial suspension but no antiserum. Successive dilutions in the remaining tubes from left to right are 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000, 1:10,000, and 1:20,000. Note agglutination in 1:10,000 but not in 1:20,000.

an adsorption, it is not always possible to remove completely the agglutinins in question.

This preferential adsorption makes possible the breakdown of the antigenic mosaic of the bacterial cell into component parts. For example, an antiserum prepared against a bacterium containing antigens A, B, and C will contain antibodies a, b, and c. If such an antiserum is absorbed by a second bacterium which contains antigens B and C, antibodies b and c will be removed leaving a intact, and the antiserum will still agglutinate its homologous bacterium. Suppose an antiserum is prepared against the second bacterium which will contain antibodies b and c. If this serum is absorbed by the bacterium containing antigens A, B, and C, it will no longer agglutinate its homologous antigen, for all the antibodies will have been removed. Clearly, then, the complete absorption of a known serum by an unknown organism does not indicate that the unknown organism is necessarily immunologically identical with that with which the known serum was prepared; to prove such immunological identity a "mirror absorption" must be carried out, i.e., each antiserum absorbed with each organism.

The agglutination test and agglutinin absorption, although used to some extent in the diagnosis of infectious disease, have been found particularly valuable in the study of the relation of bacterial species to one another and in some instances, as in the case of Salmonella (Chap. Nineteen), complex antigenic formulas have been worked out. The determination of the components of the antigenic mosaic of a bacterial species is termed antigenic analysis.

H and O agglutination. A number of bacterial species, particularly Salmonella, Proteus, and certain others, may agglutinate in two ways.

Macroscopically the H agglutination gives rise to a loose, flocculent precipitate, and upon microscopic examination it may be observed that the bacterial clumps are loose, the flagella of the microorganisms being entangled with one another. The O agglutination, on the other hand, produces a finely granular precipitate in which the individual bacterial cells are closely packed together. These types of agglutination are a consequence of the presence or absence of the flagellar, heat-labile H antigen. Immunization with the whole bacterial cell containing both flagellar and somatic antigenic components gives rise to antiserums containing both types of antibody, the H generally in high titer while the O antibody is commonly active in dilutions of less than 1:1000.

Spontaneous agglutination. Some strains of bacteria do not form stable suspensions and are said to be spontaneously agglutinable. This behavior is particularly characteristic of rough variants and, although not an immunological phenomenon, is frequently of practical importance in agglutination studies.

Cold agglutinins. In some diseases socalled cold agglutinins, which agglutinate human O group erythrocytes at O° C. but not at 37° C., are present in the serum. They are found in one-half or less of cases of primary atypical pneumonia and have limited utility in eliminating certain other infections, such as Q fever, in the diagnosis of this disease. The titer reaches a peak early in convalesence, but the agglutinin has, so far as is known, no immunological status, for it is found in other diseases such as trypanosomiasis and hemolytic anemias, and it may appear following sulfonamide therapy.

The mechanism of agglutination. The clumping of bacteria under the influence of immune serum may be taken as evidence per se that a force attracting the cells to one another is operative, at least at times. Similarly, the fact that bacteria are not in a constant state of agglutination is indicative of a force which tends to hold the cells apart from one another. The attractive or cohesive force is probably that of surface tension, i.e., the interfacial tension at the cell surface, and the repulsion that of like electrical charges, for, as pointed out previously, bacteria are negatively charged at pH's compatible with viability. On the basis of such reasoning it would appear that the balance between these opposing forces determines whether the microorganisms will form a stable suspension or whether they will clump together and settle out.

It was early observed that the presence of an electrolyte is essential to agglutination: if both immune serum and bacterial suspension are dialyzed free of salt before mixing, the cells are not agglutinated, but if a tract of salt is added to the mixture, agglutination takes place at once. This behavior, it will be seen, corresponds to that of a mixture of two colloids of opposite charge, such as gum mastic and gelatin, when the one is added in too small an amount to precipitate in the absence of salt. That the repelling effect of electrical charge is an important factor is also indicated by the agglutination of bacteria in the absence of antibody when the pH is lowered to the iso-electric point of the cells, a phenomenon termed acid agglutination. It was formerly thought that bacterial species might be sharply differentiated from one another on the basis of the pH of their acid agglutination, but this has not proved to be true.

The agglutination of bacterial cells is, however, a function not only of electrical charge on the cells but also of the cohesive forces tending to draw them together. Studies in which both potential difference (between the cells and the suspending medium) and cohesive force were directly measured have shed considerable light on the mecha-

nism of agglutination. It has been found that electrolytes in low concentration (0.01 N) affect primarily the potential, and in high concentration also decrease the cohesive force. If the cohesive force is not affected, agglutination occurs when the potential is reduced below the critical point of 15 mv. If, however, the cohesive force is decreased, the critical potential is also decreased, and, therefore, in concentrated salt solutions agglutination does not take place even though there is no measurable potential. Immune serum, presumably adsorbed on the surface of the bacterial cells, while reducing the charge somewhat appears to function by preventing the salt from decreasing the cohesive force, and agglutination occurs at the critical point of 15 mv. Furthermore, if the potential difference is reduced by electrolyte to 15 mv. or less in a bacterial suspension, the addition of immune serum raises the reduced cohesive force, and agglutination takes place. Examination of typhoid bacilli by the electron microscope in the presence of immune serum has shown that the flagella become thickened by the deposition of an antibody film approximately 21 Å thick, and the cell walls become more opaque and less definite in outline. The serum-sensitized surfaces appear to be sticky, not only for one another, but also for other particulate matter.

Serological adhesion. 45, 55 This stickiness may be in part at least the basis of the phenomenon of serological adhesion in which nonspecific particulate material tends to adhere to particulate antigen in the presence of antibody and complement. It was first observed in vivo as an adhesion of platelets to cholera vibrios, trypanosomes, and several kinds of spirochetes to provide the basis for serodiagnostic tests. It is variously known as the Levaditi phenomenon, the Laveran-Mesnil reaction, the Rieckenberg reaction, and as the Brussin or Beladung reaction in the Russian literature. What is probably the same phenomenon was later called immune adherence in a reaction in which bacteria, especially syphilis spirochetes, adhere to red blood cells in the presence of antibody and complement.

The nature of this reaction is obscure. It may be observed *in vitro* as well as *in vivo*; various kinds of particles will function in the reaction, but there are discrepancies as among red cells of varied origin, and

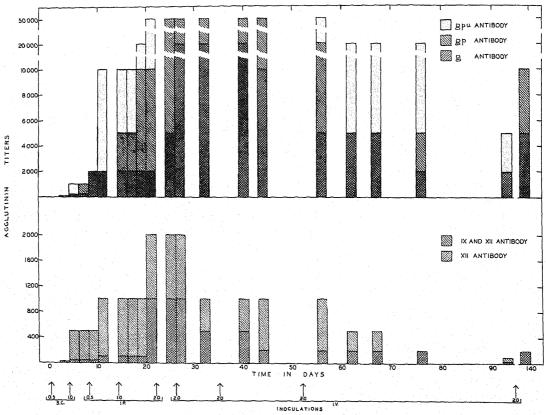


Figure 75. The differential immune response to the components of an antigenic complex as illustrated by the serum antibody response of the rabbit to immunization with Sal. enteritidis var. rostock (Sal. rostock). Agglutinin titers measured against heterologous antigens without antibody absorption. Top, antibody to flagellar antigens; bottom, antibody to somatic antigens.

adhesion has been found to occur with some bacterial antigens but not with others such as Salmonella. This reaction, using human erythrocytes as the adhering particles, has been developed by Nelson as a diagnostic test for syphilis, the *Treponema pallidum* immune adherence, or TPIA, reaction in which the spirochetes are mixed with complement, antiserum, and red cells, and the adherence of the red cells to the spirochetes is observed microscopically *in vitro*.

Blocking antigens. The presence of antigenic cell components may interfere with or prevent agglutination of bacteria by homologous antiserum. The presence of Vi antigen in typhoid bacilli, for example, renders them inagglutinable with homologous O antiserum, but they become agglutinable as the interfering antigen disappears during successive subcultures. Similarly, colon bacilli contain a thermolabile component of the envelope antigen,

the L antigen, which inhibits agglutination with O antiserum, but the bacterial suspension becomes agglutinable when this component is destroyed by boiling. This blocking effect of some antigens may be due to a spatial effect, such as steric hindrance, in preventing the primary union of antigen and antibody; or the inhibition may be one of the aggregation of sensitized cells to give agglutination in the secondary stage of the antigen-antibody reaction.

IMMUNOFLUORESCENT STAINING^{6, 8, 16, 17, 51, 54}

The labeling of immune globulin by conjugation with fluorochromes was originated by Coons in 1942. It had been established that aromatic isocyanates may be coupled with protein by a carbamide linkage with

free amino groups of the protein, commonly the ϵ -amino groups of lysine residues. This reaction is used to couple fluorochromes, most commonly fluorescein or rhodamine as their isocyanates, with immune globulin. The globulin is separated from the other serum proteins by the conventional salting out or methanol precipitation methods and reacted with the fluorochrome isocyanate, and the excess fluorochrome removed by dialysis. The antibody conjugate retains its antibody activity though some changes in other properties may occur.

Homologous antigen is specifically stained through the antibody activity of the conjugate, and may be in particulate form such as bacteria or vegetative virus, or as concentrations of soluble antigen. The application may be direct or indirect: i.e., the antigen may be stained directly with homologous antibody, or indirectly by first treating the antigen with conventional homologous antibody and then staining by the application of fluorescent antibody to the antibody globulin as a variation on the antiglobulin technique described above. In any case, the specifically stained antigen fluoresces in ultraviolet light, requiring appropriate microscopic equipment for observation. Antigen stained with fluores-

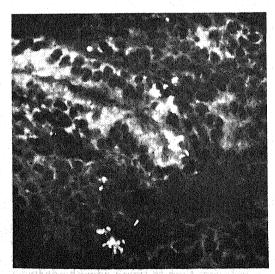


Figure 76. The demonstration of dysentery bacilli in the epithelial cells of the midportion of a villus and in the lamina propria of the guinea pig small bowel by fluorescent-antibody staining of a frozen section. The bacilli, coated with fluorescein-labeled antibody, fluoresce a brilliant pale green under ultraviolet light and appear here as white. (LaBrec.)

cein conjugates fluoresces a yellowish green color, and that stained with rhodamine conjugates a reddish orange; both tend to fade on prolonged observation. There are a number of detailed descriptions of the foregoing techniques.^{10, 18, 70}

While much less sensitive than the labeling of antigen or antibody with a radioactive isotope such as I¹³¹, the fluorescent antibody method is much more flexible and has been invaluable in the localization of antigenic substances in the tissues, especially viruses which are otherwise not directly demonstrable. It also has considerable value in the rapid identification of bacteria. 11, 70 Nonspecific fluorescence often occurs but usually may be reduced to insignificance by preliminary absorption of the antibody as, for example, with a brei of the tissue to be stained for contained homologous antigen.

ANTITOXINS

It was found by von Behring and Kitasato in 1890 that the immunity of rabbits and mice that had been immunized against tetanus was associated with the ability of the blood serum to neutralize the toxic substances produced by the tetanus bacillus. The substance in the serum which neutralized the tetanus toxin was designated by these workers as antitoxin. Subsequent investigation has shown that the animal body forms antitoxins in response to the injection of a variety of antigenic poisons, not only those of bacterial origin such as diphtheria toxin, botulinus toxin, and the like, but also against the phytotoxins and zootoxins.

The action of antitoxin may be directly demonstrated in the following way: if a fatal, or many times fatal, dose of toxin be mixed with an appropriate amount of antitoxic serum in vitro, the injection of the mixture into a susceptible animal is wholly without injurious effect; the poisonous qualities of the toxin are nullified by the immune serum. The reaction is, like all other immunological reactions, highly specific, and an antitoxin which neutralizes the homologous toxin is without effect on heterologous toxins.

The toxin-antitoxin reaction. The combination of toxin and antitoxin does not

necessitate the complete destruction of either component; neutral mixtures of toxin and antitoxin may be dissociated by treatment with hydrochloric acid, by freezing in the presence of phenol or tricresol, and, to some extent, by simple dilution. In certain cases, e.g., pyocyaneus toxin and certain snake venoms, in which the toxin is more resistant to heat than the antitoxin, the latter may be selectively destroyed, and the neutral mixture becomes toxic upon judicious heating. It appears, therefore, that a more or less loose combination of toxin and antitoxin takes place, the poisonous properties of the toxin being neutralized as long as the union persists. The rate of reaction between toxin and antitoxin, like the chemical reactions, is dependent upon temperature, concentration, character of the medium in which the reaction occurs, and similar factors. The avidity of an antitoxin for its corresponding toxin differs in different cases; the union between tetanus toxin and antitoxin, for example, takes place less rapidly than that between diphtheria toxin and antitoxin.

An understanding of the precise character of the toxin-antitoxin reaction is dependent upon the interpretation of phenomena revealed by quantitative studies. It might be expected that a given quantity of antitoxin would always neutralize a constant amount of toxin, that the neutralization would follow the law of multiple proportions. This is, however, not the case, and it appears that the amount of antitoxin required to neutralize a given quantity of toxin is dependent upon (a) the manner in which the two are mixed with one another and (b) the relation between toxicity and combining power in the particular filtrate under consideration.

In the first instance it has been observed that when an excess of toxin is added to its specific antitoxin in several portions at proper intervals of time, much more unneutralized toxin remains in the mixture than if the same quantity of toxin had been added to the same quantity of antitoxin at one time. This is known as the Danysz phenomenon. If, on the other hand, antitoxin is added to toxin in successive equal portions, it may be shown that, in general, the first portion of antitoxin neutralizes a greater portion of the toxin than the second, the second a greater than the third, etc.

In the second case it has been found that there is no constant relationship between toxicity and combining power of a toxic filtrate; toxicity slowly diminishes upon storage but combining power remains unchanged. As described elsewhere (Chap. Nine), the toxin is unstable and is slowly converted to toxoid. Both toxoid formed in this way, and that prepared by toxin inactivation with formaldehyde, retain their antigenicity and combine with antitoxin as well as stimulate the formation of antibody when used as immunizing agents. Since the proportions of toxin and toxoid are not fixed, there is only an approximate correspondence between combining power and toxicity, and the toxicity neutralized by a unit quantity of antitoxin may vary from one filtrate to another or in the same filtrate at different times from 30 to 130.

The standardization of antitoxins. quantitative evaluation of the toxin-neutralizing capacity of antitoxic serums is clearly a matter of considerable practical as well as theoretical importance. Ehrlich originally proposed as the standard unit of diphtheria antitoxin that amount which would just neutralize 100 guinea pig MLD's of toxin. As indicated above, however, variability in the relation of toxicity to combining power invalidates any standard based on toxicity; in other words, the combining power of a toxin is not a measure of its toxic qualities. On the other hand, since the combining power of a toxic filtrate remains constant within narrow limits, it is possible to establish an arbitrary standard unit upon which the relative strength of all antitoxic serums can be based. Such a standard diphtheria antitoxin was first prepared by Ehrlich and was preserved by him with all precautions against possible deterioration. A standard antitoxic serum based on Ehrlich's arbitrary standard unit is also prepared in this country by the National Institutes of Health of the United States Public Health Service, and is distributed every two months to the licensed producers of commercial serum. The unit is international, standard serums being tested from time to time under the auspices of the Expert Committee on Biological Standardization of the World Health Organization.52

Titration of neutralizing capacity. Three methods are used in the titration of diph-

theria toxin and antitoxin. The first of these is the classic method of Ehrlich in which two limits (Lat., limes) are determined by guinea pig inoculation. These are the L₀ dose of toxin, which is defined as that amount exactly neutralized by the standard unit of antitoxin, and the L+ dose, that amount of toxin which, when mixed with 1 unit of standard antitoxin, is just sufficient to kill in four days a guinea pig approximately 250 gm. in weight. With these limits established, the serum to be standardized is mixed with the toxin just titrated, and the largest amount of serum which, when mixed with the L+ dose of toxin, will produce a 100 per cent mortality in the inoculated guinea pigs is considered to contain 1 unit of diphtheria antitoxin. This method is not mathematically sound as indicated elsewhere (Chap. Nine), but the dose-response curve to the bacterial exotoxins is so steep that the procedure is practical and so persists.

A second method is based upon the observation that the intradermal injection of 1/250 to 1/500 MLD of diphtheria toxin into a guinea pig is followed by a local reaction, swelling and erythema and, with slightly larger amounts of toxin, necrosis, a phenomenon sometimes called the Römer reaction. By the use of such intradermal inoculations an Lr dose of toxin may be determined, i.e., that amount of toxin, which when mixed with 1 standard unit of antitoxin, will produce the minimal skin reaction. The serum to be standardized is mixed in varying amounts with Lr doses of toxin, and that amount of serum which gives the minimal skin reaction is considered to contain 1 unit of antitoxin. This method has the advantage of allowing the testing of a number of toxin-antitoxin mixtures in one animal but has not displaced the classic method in common usage.

Flocculation. The third method is that of toxin-antitoxin flocculation, sometimes called the Ramon flocculation, and is a precipitin reaction in which the end point is the equivalence zone. Standard antitoxin is mixed with varying quantities of toxin and the tube first, i.e., in time, showing precipitation contains one Lf dose of toxin. Varying amounts of the serum to be standardized are mixed with the Lf dose of toxin. The amount of serum in the tube first showing flocculation in this second series is con-

sidered to contain 1 unit of antitoxin. The precipitation, or flocculation, curve is, as described elsewhere (Chap. Thirteen), of the horse type in which the antigen-antibody complex is soluble in an excess of either reactant, and the Lf dose so determined is the optimal proportion zone of the reaction. This method differs from the other two in that it depends upon the combining power of a toxic filtrate rather than on toxicity. It is generally used, not as a final method of standardization, but as a preliminary to standardization by the Ehrlich method.

The interrelationships of these limits are of some interest. The L+ dose is, of course, larger than the L₀ dose and, because of the peculiarities of the toxin-antitoxin reaction, by considerably more than one MLD. The Lr dose approximates the L_0 dose, as might be expected in view of the small amount of toxin required to elicit the skin reaction. The Lf dose is generally somewhat less than any of these, since it is a measure of combining power rather than toxicity and is unaffected by differences in the proportions of toxin and toxoid. It would appear that the Lf/Lr ratio should be the same for a given toxic filtrate immediately upon standardization. It has been found, however, that this ratio differs with different antitoxic serums because of variation in the avidity of antibody.

Antibody to enzymes. 12, 68 The foregoing discussion of the toxin-antitoxin reaction applies only to the bacterial exotoxins, and diphtheria toxin in particular; e.g., tetanus toxin does not flocculate as rapidly or sharply as does diphtheria or botulinum toxin. Other toxic activities of microbial origin, such as endotoxins, are only incompletely neutralized by antibody, and still others are enzymatic in nature or closely associated with enzymatic activity. The early hypothesis that bacterial toxins might be enzymes has not been borne out in the case of the exotoxins but, in a sense, seems to receive support from theories of the nature of the action of exotoxins such as diphtheria toxin (Chap. Nine) and the enzymatic toxicities.

The way in which antitoxin serves to neutralize the activity of exotoxins in the toxin-antitoxin complex is not known. Some light may be shed on this point, and the partial neutralization of other toxicities, by indirection through the study of the inhibition of enzyme activity by antibody to the

enzyme, for the enzymatic activity represents an activity of the molecule which may or may not be related to its antigenic character.

The degree of inhibition of enzymatic activity in the presence of specific antibody is a function of amount of antibody present. The rate of inactivation, usually titrated by adding increasing amounts of antibody to a constant amount of enzyme, is at first linear, but the rate falls off, and in antibody excess a residual level of activity persists. The amount of residual activity is often related to the size of the substrate molecule, inhibition being substantially complete in the case of proteinases, collagenase, hyaluronidase, etc., and almost complete lack of inhibition is observed only rarely and when the enzyme has a low molecular weight substrate, viz., \(\beta\)-galactosidase, tyrosinase, catalase, etc.

The inactivation may be only apparent in that aggregation of the enzyme in the antigen-antibody precipitate reduces its surface available to the substrate; such apparent inactivation is observed, for instance, with catalase. True inactivation appears to be a matter of steric hindrance, small substrate molecules penetrating between molecules of adsorbed antibody to the active sites on the surface of the enzyme, while large substrate molecules do not. This inference is not only supported by the general, though not invariable, relation of inhibition to size of the substrate molecule, but also by the varied consequences of the order and amount in which antibody and substrate are added to the enzyme-i.e., antibody will not displace substrate—and by kinetic studies which indicate that antibody and substrate do not compete for the same re-

It appears, then, that the site of enzymatic activity and that of antigenic specificity on the enzyme are not necessarily identical. In support of such an inference, cross-reactions between functionally similar enzymes from widely different sources seldom occur, but they do occur between enzymes of closely related origin, and the latter is apparently attributable to antigenic relationships between the proteins rather than the prosthetic groups of the enzymes. While it seems clear that antigenicity and enzymatic activity need not be identical for inhibition of activity

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by antibody, it has not yet been possible to show unequivocally that the two are invariably not identical.

The analogy to the neutralization of toxin by antitoxin will be apparent at this point. The occurrence, or preparation, of toxoids from exotoxins, lacking toxicity but having unimpaired antigenicity, may be taken to suggest that the toxic portion of the molecule may be independent of its antigenic specificity. Similarly, the endotoxins are often said to be poor antigens, since toxicity persists in the presence of excess antibody. They are, in fact, excellent antigens as assaved by the titer of, for example, precipitating antibodies they produce, but the antigen-antibody precipitate at the equivalence zone is toxic although no toxicity is detectable in the supernatant. In a sense, then, the neutralization of toxin by antibody may be only incidental to the antigen-antibody reaction.

LYSINS

It was observed by Nuttall in 1888 that freshly drawn and defibrinated blood, or serum, is markedly bactericidal for many kinds of bacteria and that this property is heat-labile and destroyed by holding at 55 to 56° C. for 30 minutes. Such heated blood or serum is said to be inactivated. This bactericidal effect is a consequence of the presence of natural antibody (Chap. Fifteen) and possibly also properdin (Chap. Nine) in the blood or serum, acting in conjunction with the thermolabile activity known as complement. The antibody concerned is cytotoxic or cytolytic and, because the lytic effect is the one most commonly made use of experimentally, such antibodies are lysins. In functioning as a lysin, the antibody mediates the effect of complement to give a secondary stage of the antigenantibody reaction that is clearly set apart from the primary union or extensions of it.

Pfeiffer phenomenon. One of the first demonstrations of the phenomenon of immune lysis was that by Pfeiffer in 1894 of the lysis of the cholera vibrio in the intraperitoneal cavity of the immune guinea pig. A heavy suspension of the vibrios is injected and samples of peritoneal fluid withdrawn from time to time and examined microscopi-

cally. The vibrios first lose their motility, swell up, and crumble into small fragments, and dissolution of even the fragments follows so that no trace of the bacterial cell remains visible. This phenomenon may also be observed *in vitro* when the vibrios are mixed with antibody and complement.

Cholera vibrios and related microorganisms are relatively fragile, more so than most kinds of bacteria. Immune bacteriolysis may be observed with these microorganisms, and with certain others; for instance, some portion of, but not all, typhoid bacilli so treated will lyse. While lysozyme contained in serum, including complement. and in other body fluids is considered to play a part in the immune bactericidal and bacteriolytic reactions, a residual effect remains after the removal of lysozyme from the system.29 In the case of the cholera vibrio, it has been shown that the cell wall is depolymerized in the presence of the antibody-complement complex in the absence of lysozyme to produce protoplasts which are viable but die because of their osmotic fragility: i.e., the bacteriocidal effect could be completely prevented by the addition of protective substances.25 The bactericidal effect, however, may occur without visible lysis, perhaps by alteration of permeability and loss of cell constituents, and it may be shown by appropriate serological methods that the cellular antigen has combined with both antibody and complement.⁵³

Hemolysis. The lysis of bacteria by an immune serum is not a unique reaction in which only bacterial cells may play a part; bacteriolysis is, rather, a special case of a general phenomenon, for immunization with a variety of cells results in the production of cytolytic serums. Of these, erythrocytes have been by far the most widely studied, for the lysis of these cells, hemolysis, is readily apparent in the test tube; the red opacity of the cell suspension changes to the clear red solution of hemoglobin as lysis proceeds. The stroma is not dissolved but, upon examination, appears misshapen. First demonstrated by Bordet in 1898, hemolysis has been particularly useful not only as a type of lytic reaction peculiarly suitable for laboratory manipulation, but also as an indicator of antigenantibody combination when visible lysis does not or cannot take place. Such immune hemolysins, *i.e.*, those formed by the animal body in response to the injection of erythrocytes, are quite unrelated to the hemotoxins or hemolysins formed by microorganisms.

Complement.^{30, 48, 58, 66} The heat-labile activity participating in the lytic reaction is a constituent of normal serum, and its presence is unrelated to the immune response; *i.e.*, it does not increase in amount during immunization. This activity was called alexin by Bordet, in a restriction of the term as originally applied by Buchner to the undifferentiated heat-labile bactericidal action of blood, and complement by Ehrlich; the latter term is by far the more widely used.

Its status as a normal serum component is clearly indicated by the fact that lytic or bactericidal activity of freshly drawn serum, which has been destroyed by heat inactivation, is promptly restored by the addition of fresh normal unheated serum. There is little species specificity in complement; that from guinea pig serum, for example, may participate in the lysis of beef erythrocytes in the presence of antiserum prepared in the chicken. Some quantitative differences are apparent, for a serum which may be actively complementary in some reactions may be relatively inactive in other combinations, and the total activity is a function of the relative amounts of the various components of complement (see below) that may be present in a given serum.

Properties of complement. The property of inactivation by heat has been referred to above. Complement is also inactivated by shaking, but in neither case is the inactivation completely irreversible, for some activity may be regained on standing. In this respect complement behaves as a typical hydrophilic colloid which can be made to aggregate by physical forces but shows a tendency to spontaneous dispersion and restoration to the original state. The activity disappears upon standing-rapidly at room temperature (two or three hours) and more slowly in the icebox, where it may be preserved for three or four days. It is quite stable at very low temperatures in frozen form, and most of the activity persists after lyophilizing, and in lyophilized form it may be stored in a refrigerator for some months. Complement is irreversibly destroyed by strong acids or alkalis and reversibly inactivated by ions such as Mg. Ca. Ba. Sr. and SO₄ or by hypertonic salt solutions. In this connection it is of some interest that complement which is inactivated by raising the salt concentration to 5 to 10 per cent may be preserved at low temperatures in this form for several weeks, the activity being regained upon dilution with distilled water. These properties and some others, such as ready adsorption on surfaces, suggest a close relationship between complement and the enzymes; it has also been pointed out that there are remarkable resemblances to certain compounds of protein with soaps and lipids. An association of complement with the blood-clotting mechanism is suggested by the observation that many substances have both anticoagulant and anticomplementary activity, and that there is a close correlation between thromboplastic and complement-fixing power of tissue extracts and serums. It seems clear, however, that complement is not identical with prothrombin, and available evidence suggests that inactivation of complement of plasma blocks the conversion of prothrombin to thrombin. The nature of the lytic action of complement is as yet, however, purely speculative.

Complement is apparently rapidly formed in the intact animal; it has been found that, following apparently complete decomplementation of the guinea pig by the inoculation of egg albumin and homologous rabbit antiserum, appreciable complement titer is detectable at eight hours and by 18 hours

has returned to normal.

Nature of complement. Complement activity is intimately associated with the serum proteins and is inactivated by enzymatic digestion. The activity is not a property of a single substance, but of more than one differentiable substance. For example, when electrolyte is removed from serum by dialysis against distilled water, the euglobulin fraction of the serum proteins is precipitated, and a part of complement activity is present in this fraction and a part in the supernatant. Neither is active alone, but when they are recombined, activity is restored. The component present in the euglobulin fraction will unite with an antigen-antibody complex but does not produce lysis, while the soluble fraction will not combine with the antigen-antibody complex but with the antigen-antibody complex plus the euglobulin fraction. Because of this, the euglobulin fraction is known as the midpiece of complement, and the other fraction as the end-piece. By a combination of various properties, complement, customarily designated C', is separable into four fractions. These are:

C'₁: This fraction is the mid-piece described above which is precipitated by dialysis, or by bubbling CO₂ through serum diluted 1:10 with distilled water. It is heat-labile and is a euglobulin containing carbohydrate.

 C_2 : This fraction is the end-piece and is also heatlabile. It is a mucoglobulin containing 10 per cent

carbohydrate.

C₃: This is a heat-stable fraction which is inactivated by treatment with cobra venom, yeast, or zymosan but is not inactivated by treatment with ammonia or pri-

mary amines.

 C_4 : This is a heat-stable fraction which is inactivated by treatment with dilute ammonia or primary amines such as hydrazine, or by shaking with chloroform or ether, but is stable to treatment with cobra venom, yeast, and zymosan. It appears to be a carbohydrate portion of the mucoglobulin fraction containing C_2 , whose carbonyl groups are attacked by ammonia.

There is evidence that C'_{3} may be resolved into two factors C'_{3a} and C'_{3b} , 64 , 89 which may participate in successive steps in the hemolysis sequence (see be-

low). 3, 63

These components of complement are found in various animals but in differing proportions. For example, C'2 and C'4 are present in only small amount in the serum of the horse, mouse, cow, and sheep; sheep serum is also deficient in C'3; and C'2 and C'3 are present in smaller amount than the other components in guinea pig and human serums. Since all four components are required for lysis, the complement titer of a serum is limited by the fraction occurring in smallest amount. Of readily available animal serums, that of the guinea pig usually shows the highest titer and is commonly used as a source of complement in serological reactions, either fresh or in lyophilized form.

The components of complement are titrated by using complement deficient in the fraction to be titrated. Such preparations are designated R'; and R'₁ is deficient in C'_1 , R'_2 in C'_2 , etc. The components tend to be functionally interchangeable to a considerable degree, as between human and guinea pig serum. On the basis of analyses of the complex, about 200 to 400 μ g. of protein per milliliter of serum combines with the antigen-antibody complex, representing the complement exclusive of C'_3

which does not combine, and the bulk of that which combines appears to be C'_1 .

Mechanism of the lytic reaction. It is already apparent from the foregoing discussion that the lytic reaction involves three components, the antigen, the antibody. and complement. This is illustrated in the accompanying schematic form for the socalled hemolytic system, i.e., that in which the antigen is the erythrocyte. The three components of the system do not react with one another at random. The antigenantibody reaction occurs first, and then complement, which does not react with either antigen or antibody alone, reacts with the complex. This may not hold true invariably, for there is evidence⁷¹ that C'₄ and C'₂ may combine with antibody alone. This may be shown in a number of ways. For example, if either antigen or antibody alone is mixed with complement, the complement activity remains free as demonstrable by appropriate titration methods (see below). Or, the antigen-antibody reaction occurs rapidly, even at O° C., but complement unites with the complex only slowly at this temperature. 50

It appears, therefore, that in immune lysis the antibody is not responsible for lysis, but rather mediates the action of complement as the lytic agent. Consequently, within this framework, antibody has been called the sensitizer, or substance sensibilisatrice, by Bordet and the French workers, and amboceptor by Ehrlich. Relatively small amounts of both antibody and complement suffice for lysis. Heidelberger has calculated that about 1500 molecules of rabbit antibody and 25,000 molecules of guinea pig complement are enough to lyse a single sheep erythrocyte. While figures such as these are no more than approximations, it is clear that only a very small part of the surface of the red cell need be affected to result in lysis.

The amount of antibody present is not a critical factor, but in regions of great excess of either antigen or antibody, lysis does not occur on the addition of complement. It is obvious that lysis will not occur if there is not enough antibody present. The failure of lysis in the presence of excess antibody is the phenomenon of complement deviation, or the Neisser-Wechsberg phenomenon, and is analogous to the prozone phenomenon in other serological reactions. The sensitizing action of antibody in the hemolytic system has a certain enzymatic character in that, in the presence of excess complement, antibody is transferred from one red cell to another, and lysis continues to occur slowly over a relatively long period.49

It is a matter of considerable interest that immune lysis may be passive. If, for example, a haptene is coupled with the erythrocyte by diazotization, or soluble antigen is adsorbed onto the surface of the red cell as in passive hemagglutination, such red cells are lysed by complement in the presence of antibody to the haptene or adsorbed antigen. Apparently, then, the combination of complement with the antigen-antibody complex in proximity to the surface of the red cell suffices to produce lysis even though the structure of the red cell is not directly involved in the initial antigen-antibody reaction. The way in which lysis occurs, in the case of the erythrocyte as leakage of the hemoglobin, is essentially unknown. On the basis of the dependence of inactivation of C'2 and C'4 by plasmin on the presence of C'₁ and calcium ions, it has been suggested46 that C'1 is a proenzyme which can be converted to an active enzyme by plasmin and the antigen-antibody complex, the latter serving as a catalyst in the activation. It is now definitely indicated the C'1 is an esterase precursor which is activated during hemolysis, but its esterase activity appears to be independent of its activity as a com-

erythrocytes + unheated immune serum = hemolysis (antigen) (complement or alexin

⁺ amboceptor or sensitizer)

erythrocytes + heated immune serum = no hemolysis (antigen) (amboceptor or sensitizer)

erythrocytes + heated immune serum + unheated normal serum = hemolysis (antigen) (amboceptor or sensitizer) (complement or alexin)

ponent of complement.³³ In this connection it is of interest that red cells may be sensitized to the lytic action of complement by treatment with substances such as colloidal silicic acid or tannic acid.

The reaction of complement with the sensitized cell is dependent upon the presence of both magnesium and calcium ions. In the case of lysis of sheep erythrocytes by Forssman antibody (it will be recalled that the sheep red cell contains Forssman antigen), the reaction with complement occurs in an orderly way.5 The sequence of three reactions of the several components of complement with the antigen-antibody complex, the first of which requires calcium, and the second magnesium, ions. Using the symbols E = erythrocyte, and A = antibody, this sequence is represented by 1 below. Available evidence suggests that the last step indicated may, in fact, be resolved into at least three separate reactions.24

Complement fixation.⁵⁷ Complement reacts with any antigen-antibody complex, including the precipitate from the precipitin reaction, toxin-antitoxin floccules, agglutinated bacteria, etc. When this reaction occurs, complement activity disappears in that it is no longer demonstrable in free solution, and the complement is said to be fixed. This is the complement fixation reaction or Bordet-Gengou phenomenon.

In systems in which the antigen is other than a lysable cell, there is no overt evidence that complement fixation has occurred, but the presence or absence of free, unfixed complement may be shown by the addition of an indicator system. This is a hemolytic system consisting of erythrocytes together with homologous antibody, and usually is sheep erythrocytes and homologous antiserum prepared in the rabbit. This provides the basis of the serological reaction known as the complement fixation test or reaction 2 below.

In this, as in other serological reactions, either the antigen or antibody may be unknown. If a known antiserum is used, an unknown microorganism or other antigen may be identified, and, conversely, serum may be titrated for antibody to a known antigen. This kind of serological reaction is especially useful when the antigen, known or unknown, particulate or soluble, may be difficult to prepare or obtain in purified form or in reasonably large amount, and is widely applied to viral and rickettsial antigens and antibodies.²⁶ It has been most familiar as the Wassermann test applied in the serodiagnosis of syphilis.

The question which is asked in the complement fixation reaction is the same as that asked in other serological reactions; viz., do the antigen and antibody react? If union has occurred, complement is fixed, and its activity is no longer demonstrable in the reaction mixture by the hemolytic indicator system, and hemolysis does not occur. Conversely, if the antigen and antibody do not react, there is no antigen-antibody complex to fix complement, and hemolysis occurs in the indicator system. The specificity of this serological reaction lies, then, in the initial reaction between antigen and antibody, and the indicator system is nonspecific. For example, while the sheep erythrocyte and its homologous rabbit antibody are commonly used as the indicator system, the

Ť.

E + A
$$\longrightarrow$$
 EA (the initial antigen-antibody reaction)

EA + C'₂ + C'₄ $\xrightarrow{\text{Ca}^{++}}$ EAC'_{1,4}

EAC'_{1,4} + C'₂ $\xrightarrow{\text{EAC'}_{1,4,2}}$ EAC'_{1,4,2}

EAC'_{1,4,2} + C'_{3b} $\xrightarrow{\text{EAC'}_{1,4,2,3b}}$ EAC'_{1,4,2,3b}

EAC'_{1,4,2,3b} + C'_{3a} $\xrightarrow{\text{EAC'}_{1,4,2,3b}}$ E* + inactive product

E* $\xrightarrow{\text{stroma}}$ stroma + hemoglobin

2.

specificity of the complement fixation reaction would be unaffected if the rabbit erythrocyte and its homologous antibody prepared by immunization of the chicken were used.

The complement fixation reaction is technically more complex to carry out than many of the other serological reactions. It must be shown, for example, that neither the antigen nor the antibody is anticomplementary as used, i.e., that neither alone reduces the activity of added complement. Similarly, the amount of complement to be added must be determined by preliminary titration, and any residual complement activity in the serum to be tested must be removed by heat inactivation; too much or too little complement in the system will give false-negative or false-positive reactions respectively. In addition, the reagents must be added in the proper order. The antigen and antibody are allowed to react in the presence of complement for a sufficient time to allow the antigen-antibody reaction to occur and for the complex to react with, and fix, the complement. Then the indicator hemolytic system, red cells and homologous antibody, the latter also previously titrated, is added and allowed to react with any complement that may be present. Representative protocols for the complement fixation reaction and the required reagent titrations are given elsewhere (Chap. Two).

Quantitation. 42 The amount of antibody present or, conversely, the ability of the antigen to react with the antibody, may be approximated by carrying out the complement fixation reaction with serial dilutions of the antiserum. It may be made more precisely quantitative by determining how much complement is fixed by the antigen-antibody complex. This is carried out by adding a precisely known excess of complement and backtitrating the unfixed complement with the hemolytic system.

The reaction is read as the amount of hemolysis and is inverse, in that hemolysis is indicative of a negative antigen-antibody reaction. The end point may be taken as complete hemolysis, designated as ++++, and varying degrees of hemolysis as +, ++, or +++. The inverse relationship appears when no hemolysis is recorded as a ++++ antigen-antibody reaction, + hemolysis as +++ antigen-antibody reaction, etc. While

this relatively crude criterion is sufficient for many purposes, the end point of complete hemolysis is subject to the same fallacy as a 100 per cent killing of microorganisms by a disinfectant (Chap. Six) or a 100 per cent mortality as an assay of virulence of toxicity (Chap. Nine); i.e., the sensitivity of erythrocytes to lysis is approximately normally distributed and 100 per cent lysis is a limit approached asymptotically. A more precise measure of hemolysis is, then, an interpolated 50 per cent end point from observed fractional hemolysis assayed photometrically.

OPSONINS

If a mixture of polymorphonuclear leucocytes and bacteria or other particulate matter is incubated for a time, it will be found on microscopic examination that a number of the leucocytes have ingested the foreign particles. Few if any particles will be ingested if the mixture is prepared in physiological salt solution, a considerable number if the fluid is normal serum. In the case of bacteria, great numbers of the microorganisms will be found packed into the leucocytes when the two are suspended in the specific immune serum. The antibodies present in the immune serum which so remarkably stimulate this engulfment by body cells are designated bacteriotropins, a term not in common use, or opsonins. The term

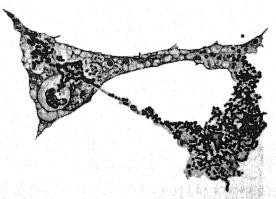


Figure 77. Phagocytosis of pneumococci by culture macrophages. The lightly staining pneumococci are degenerating. Hematoxylin and eosin-azure II. \times 1200. (Zuckerman.)

opsonin was originally used to designate the activity of normal serum; hence the antibodies in the immune animal are sometimes called immune opsonins. The cells which ingest such particulate matter are termed phagocytes. This property is not confined to the polymorphonuclear leucocytes or heterophils, although these have been most widely used in in vitro experiments because of their availability, but is also present in mononuclear phagocytes, circulating and fixed tissue cells, or histiocytes, demonstrable in the latter by, for example, perfusion of isolated organs as well as in vivo.39 These cells and their role in immunity are discussed in the following chapter.

The opsonic index. A quantitative estimate of opsonin present in a given immune serum may be made by comparing the number of bacteria ingested by normal leucocytes in normal serum with the number ingested by normal leucocytes in the immune serum. The appropriate mixtures are prepared and incubated in capillary tubes, smears made and stained, and the bacteria engulfed by an arbitrary number (usually 50 or 100) of leucocytes counted. The average number of bacteria per leucocyte, or phagocytic index, is determined for the normal and immune serums, and the ratio of the phagocytic index of the immune serum to that of the normal serum is termed the opsonic index.

The opsonins may be estimated also by the dilution method; specimens are prepared in the usual way, except that the normal and patient's serums are diluted with saline or Ringer's solution. One mixture is made with salt solution or Ringer's solution to determine the degree of spontaneous phagocytosis. The dilution of serum which gives the same amount of phagocytosis as a mixture without serum is taken as the end point.

The factors influencing phagocytosis.³⁷ The process of phagocytosis is markedly influenced by environmental factors and the nature of the bacterium and leucocytes used, as well as by the amount of opsonin present. Departures from a neutral or very slightly acid reaction or from isotonicity, and the presence of certain ions, notably the citrate radical, depress the degree of phagocytosis. The last is of practical significance in that it contraindicates the use of citrated blood.

The presence of calcium, on the other hand, may restore phagocytic power to leucocytes which have been allowed to stand in isotonic salt solution for a number of hours, a point of some interest in connection with the apparent intimate relation between this ion and cell division and the ability of ameboid cells to form new surfaces.

The nature of the bacterium to be ingested is of considerable importance; in general, virulent forms are relatively resistant to phagocytosis. It is not unlikely that this resistance is associated with the presence of a capsule; in the case of the pneumococcus, for example, not only are the smooth, encapsulated forms highly resistant to phagocytosis, but the presence of capsular polysaccharide markedly inhibits phagocytosis by an immune serum, 35, 67 presumably through combination with the antibody. Bacteriophage renders bacteria considerably more sensitive to phagocytosis.

The phagocytic power or activity of the leucocytes is subject to considerable variation independent of variation in the opsonic content of the blood. This inherent phagocytic power of the leucocytes varies, with respect to certain bacteria at least, even in persons apparently in perfect health. In the child at birth the leucocytes are somewhat less active phagocytically than in the adult; they grow less active for a few months, and then more active, reaching the adult standard for streptococci, pneumococci, and staphylococci about the third year. In pneumonia, scarlet fever, and other conditions in which there is acute leucocytosis when the outlook is favorable, the phagocytic power of leucocytes has been found to be greater than normal for the specific bacteria. The increase in activity in such cases may be due to the predominance of young leucocytes.

The process of phagocytosis. The mechanism of ingestion is essentially one of the interplay of interfacial forces. Low interfacial tension of the bacteria against the leucocytes—and high interfacial tension against the medium—favors ingestion. The immune opsonins, and to some extent the "normal" opsonins, apparently form a surface deposit on the bacterial cells which promotes phagocytosis by altering the interfacial tension in this manner. In addition to surface tension, other factors such as the viscosity of the phagocytic cell substance enter into this phenomenon. Physical forces

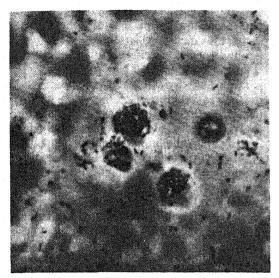


Figure 78. The phagocytosis of typhoid bacilli by leucocytes in whole blood. Note the enormous numbers ingested by the white cells and the bacilli lying free. Hastings' stain; \times 1200.

are not the sole controlling factors, however, for increased oxygen consumption accompanies the process of ingestion, the rise beginning at once, reaching a maximum value twice that at the start in about 15 minutes and persisting for 90 to 150 minutes. It has been suggested that the accumulation of hydrogen peroxide may contribute to the killing of phagocytosed bacteria.⁴⁰

It is of some interest that bacterial cells may be artificially "opsonized," i.e., made more readily phagocytable, by treatment with iron ammonium alum, chrome alum, protamine sulfate, or gallotannic acid. The effect is reversed by treatment with oxalate, but reversion is very difficult when the cells are sensitized with immune opsonin. The significance of such observations in immune opsonization and phagocytosis is not clear.

Phagocytosis may occur quite as readily in the absence of antibody as in its presence, provided that it occurs on a suitable surface; most body tissues provide such surfaces, viz., the phagocytosis of pneumococci on the alveolar surfaces of the lungs. This is the phenomenon of surface phagocytosis.⁷³

The fate of ingested bacteria. Following phagocytosis many, but not all, species of bacteria may be observed to undergo a process of dissolution, with swelling, granulation, and fragmentation appearing as successive stages in their destruction. Although it was early supposed that intracellular

digestion was no more than intracellular lysis through the agency of the immune lysin and complement which would have occurred whether or not phagocytosis took place, it now seems probable that the two processes are essentially different.

Bacteria are no exception to the rule that living organisms are not subject to attack by digestive enzymes, and the question arises whether death is a necessary preliminary to ingestion or whether it may occur within the phagocyte. It is probable that the viability of the microorganism is not an important factor in phagocytosis; in most cases the cell may be engulfed whether it is living or dead. In the case of those bacteria which are destroyed within the leucocyte, killing is necessary before digestion can take place.

Bactericidal agents are present within the phagocytic cells, but are not equally effective on all kinds of microorganisms; in some instances at least their activity appears to be potentiated by antibody.28,65 Such substances are designated leukins in the older literature; one, or a group, of these has been studied intensively and named phagocytin.³⁶ Others appear to be lysozymelike in nature. 62 The efficacy of the killing process in the case of susceptible microorganisms is illustrated in observations⁷² on avirulent streptococci, more than 90 per cent of which are destroyed in 15 minutes within the phagocytic cell. When these substances are not effective, the infected phagocytic cells serve to protect the microorganism from antibody²⁸ and may serve to disseminate the infection. Influenza virus, for example, is phagocytosed but remains infective for extended periods,7,34,43 and in general virulent bacteria such as streptococci tend to persist and may destroy the phagocyte.

Leucocytes have been shown to contain a variety of digestive enzymes, including proteases, lipases, and various carbohydratesplitting ferments which are presumably responsible for the actual dissolution of the engulfed particle.

The nature of the opsonic activity. The opsonins are true antibodies in that they are increased by immunization and exhibit the specificity characteristic of the immune antibodies. The activity is, like that of the lytic serums, made up of two components, one thermostable and the other thermolabile. The thermolabile component is pres-

ent in normal serum, and the reduced opsonic powers of an inactivated immune serum may be restored by the addition of a small amount of normal serum. In this and other respects the thermolabile component of opsonin strikingly resembles complement and in the past has been assumed by many to be identical with this component of the lytic system. In general, however, it has not been possible to show any constant relation between the components of complement and opsonization. For example, while the fourth component of complement has been reported to be necessary to the lytic reaction but not required for opsonization, it has also been observed that the thermolabile opsonin of the human serum is identical with C'1, C'2, and C'4 in combination but not separately or in any combination of two. It appears, then, that the complement and the thermolabile element of opsonin, while resembling each other closely in many respects, may not be regarded as identical.

PROTECTIVE AND NEUTRALIZING ANTIBODIES

When an antigen is a pathogenic microorganism, or a toxic product of a microorganism, antibody to it may protect a susceptible animal against infection or the effects of a toxic substance. Assay of this kind of antibody activity has been referred to earlier in connection with the standardization of antitoxins but is more widely applicable than indicated there.

The measurement of such antibody activity is dependent upon establishing a point of reference, such as the LD_{50} dose of microorganisms or toxic substance in the normal animal, and comparing with it a similar value obtained in the presence of antibody. In practice the challenge inoculum is ordinarily a multiple of the LD_{50} and, for sound mathematical reasons, is held constant while the amount of antibody is varied.

The observed protective or neutralizing effect may be attributed to opsonic, lytic, antitoxic, and other antibody activities, and does not distinguish among them. At the same time, it measures the contribution of the antibody, and inferentially the antigen preparation, to effective immunity and thereby differs from the *in vitro* serological reactions. Such tests of antibody activity are

of two general kinds, the protection test and the neutralization test, and the antibody activity so measured is said to be protective antibody or neutralizing antibody.

Protection tests. The protection test is in turn subdivided into two kinds of tests, the active protection test and the passive protection test, depending upon whether the test animal forms its own antibody in response to the inoculation of an antigen or is the passive recipient of preformed antibody from another animal.

Active protection and immunogenic potency. The active protection test is a test of immunogenic potency of the antigen. It is carried out by inoculating groups of animals with serial dilutions of antigen, or a number, usually a fairly large number, of animals with a specified dose of antigen. The former is required for most kinds of experimental studies, and the latter is often used for the routine assay of the immunogenic potency of vaccines prepared and distributed commercially.

After a time, such as one to three weeks, during which the immune response develops, the animals are challenged with some standard dose, say 1000 LD_{50} , of the virulent microorganism of the toxin. The protection conferred by immunization is measured as the amount of antigen required to protect 50 per cent, or some other specified portion, of the animals inoculated.

Since the details of this and other kinds of protection and neutralization tests are determined by the kind of microorganism or toxin used for immunization and subsequent challenge, such tests can be described in only general terms. At the same time, two points must be taken into consideration in tests of this kind. First, the susceptibility of the normal experimental animal, commonly the mouse, should be known to be reasonably constant, and animals of at least pen-bred homogeneity are usually required. Second, the microorganism must have relatively high virulence for the experimental animal for, when large doses must be used, those of living microorganisms required to kill may approach the toxicity level, i.e., the lethal dose of killed microorganisms. This is generally true, for instance, in the case of the enteric bacilli, the gonococcus. the meningococcus, and certain other kinds of bacteria. The relative virulence of the microorganism thus affects the level of protection found; usually very high titer of protective antibody, of active or passive origin, is not observed when the microorganism is relatively avirulent.

Further, the relation of the experimental infection to the naturally occurring human disease needs to be taken into consideration when such experimentally determined immunogenic potency is used to characterize antigens intended for human use. For example, the virulence of the cholera vibrio for the mouse is very low, but may be greatly increased by suspending the bacteria in mucin to give a fatal fulminating bacteremic infection on intraperitoneal inoculation with perhaps 100 vibrios. The usual test for immunogenic potency of cholera vaccines is carried out in the mouse, but it is not clear what relation the challenge infection may have to the naturally occurring human infection in which the microorganisms are confined, for all practical purposes, to the lumen of the bowel and, therefore, to what extent immunogenic potency so measured is applicable to man. Similarly, the challenge inoculum of pertussis bacilli for testing active protection in mice is given intracerebrally. The infection would seem to be quite unrelated to whooping cough in man, but it is significant that protection of mice against infection by this route parallels that against infection by the intranasal route which produces an infection in mice similar to that observed in man.

Passive protection. In the passive protection test, antiserum is tested for protective antibody by parenteral inoculation of groups of animals with graded doses in constant volume. Some time may be allowed to elapse between the administration of antiserum and challenge inoculation, six to 24 hours, so that the antibody is absorbed by the tissues of the animal. Or, if given nearly simultaneously, antiserum and challenge inoculation may be given by different routes, such as subcutaneously and intraperitoneally respectively. In any case, the passively immunized animals are challenged with some standard dose of the infectious agent or its products. The LD₅₀ dose is interpolated in the usual way and, by comparison with the LD₅₀ dose in the normal animal and the amount of antiserum required to protect 50 per cent of the immunized animals, the number of LD₅₀ doses protected against by 1 ml. of antiserum, or its protective antibody titer, is readily calculated.

Neutralization tests. The neutralization

test is usually used in the neutralization of toxin by antitoxin and for the titration of antibody concerned in immunity to viral infections.23,47 It differs from the protection test in that varied amounts of antiserum are mixed with a standard dose of microorganisms or toxin, allowed to stand for a period, such as 30 minutes, to permit the antibody present to react with the antigen, and the mixture is then titrated for infectivity or toxicity. The assay may, of course, be carried out in the intact animal, but it is also applicable to tissue culture or egg culture while the protection type of test is not. The assay criterion is the death, or other effect, of 50 per cent of animals, tissue cultures, egg cultures, etc., and the titer of neutralizing antibody is calculated in the same way that protective antibody titer is calculated in the passive protection test.

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Chapter Fifteen

THE IMMUNE STATE

Immunity to disease, or effective immunity operative in the specific immune state, is to be distinguished from the immune response in the technical sense on the one hand, and from resistance, sometimes called natural immunity or innate immunity, on the other. In the first instance, not all antigens provoke the formation of protective antibody. This is obvious in the case of antigens, such as ovalbumin, which are unrelated to pathogenic microorganisms or their products. Further, as implied elsewhere (Chap. Twelve), not all the components of the complex of antigens making up the antigenic structure of a microorganism are concerned in effective immunity to the disease which it causes. Among the enteric bacilli, for example, antibody to the heatlabile antigens is apparently quite unrelated to effective immunity, and only antibody to the heat-stable antigens is protective.

Distinction between specific acquired immunity to disease and resistance or natural immunity is usually made on the basis of specificity; *i.e.*, acquired immunity is functional only against a specific microorganism, or sometimes a group of immuno-

logically related microorganisms, while in natural immunity there is no distinction on the basis of antigenic specificity. The separation of acquired and natural immunity often cannot be made as sharply as implied. It is complicated by the common, practically universal, occurrence of so-called natural antibody, and the cellular response of acquired immunity differs quantitatively rather than qualitatively from that occurring in the normal animal.

The state of immunity to disease is separable into two parts: that which is primarily dependent upon the presence and activity of circulating antibody, humoral immunity; and cellular immunity, which is a function of the activity of phagocytic cells and the cellular response in the processes of inflammation. These parts also merge with one another, most obviously in the opsonic activity of antibody, in the function of cells in the synthesis of antibody in response to antigenic stimulus, and in the predominantly cell and tissue response of hypersensitivity mediated by antibody. Nevertheless, such a separation is useful for purposes of discussion.

Humoral Immunity

The appearance of circulating antibody during recovery from infectious disease, and its association with the refractory state of specific acquired immunity, suggests that it may be causally related to effective immunity. The significance of antibody to effective immunity is firmly established by the kind of experimental evidence which provides the basis of the passive protection and neutralization tests (Chap. Fourteen). Less direct, but highly suggestive, evidence

of the significance of circulating antibody in effective immunity is the high susceptibility to infection of individuals who fail to form γ-globulin. Congenital agammaglobulinemia, which may have a hereditary basis, has been observed in a number of instances³³ associated with susceptibility to generalized vaccinia and pyogenic infection. In general, it may be stated categorically that, so far as is known, acquired effective immunity rests upon an antigen-antibody basis.

Antitoxic immunity. The function of various kinds of antibody activity in effective immunity is a matter that becomes partly speculative. As in the case of virulence of pathogenic microorganisms when virulence is attributable to essentially a single factor, such as toxigenicity and the pharmacological activity of the bacterial exotoxins, the nature of effective immunity to such a disease is equally clear. In tetanus and diphtheria, for example, the single significant factor in acquired immunity is antitoxin, and antibody to the cell substance of the causative microorganism is, at most, of only minor importance.

In the case of other soluble toxic products of pathogenic microorganisms, differentiation of the effective from the plausible function of antibody may be resolved to some extent by the protection test. The gaseous gangrene microorganism, Clostridium welchii type A, for example, produces three welldefined toxic products: the hemolytic α and θ toxins, and the κ toxin or collagenase. It might be supposed that collagenase activity would be a highly significant factor in a disease characterized by extensive tissue destruction and that antibody to it would be functional in effective immunity. This has not proved to be true, for antibody to the α toxin is protective while those to the other two toxins are not.

Or, antibody may affect the character of the disease but not otherwise materially modify its severity or prevent it. Scarlet fever is a clinical entity only because of the activity of the erythrogenic toxin, and antibody to this toxin prevents the appearance of the scarlatiniform rash, but contributes little or nothing to immunity to infection with the causative streptococci.

Antibacterial activity. The function of antibody having other than antitoxic activity is less clear. The phagocytosis of microorganisms is a highly significant feature of immunity to infections with a variety of bacteria, such as the staphylococci, and it is highly probable that the opsonic activity of antibody plays an important part in effective immunity. In some instances, though, simple phagocytosis mediated, or accelerated, by circulating antibody may serve to protect the microorganism or even facilitate the spread of infection. Brucellosis, for example, persists in spite of high circulating antibody titer, and the inadequacy of the

immune response, together with the limited effectiveness of chemotherapeutic drugs in this disease, is thought to be a result of persistence of the microorganism within host cells.

Agglutinins and precipitins would appear on the surface to serve no useful purpose in the defenses of the immune animal; for example, bacteria are not killed by agglutination. The clumping of bacteria and the precipitation of soluble foreign protein, however, considerably facilitate the process of phagocytosis, in that many bacteria may be engulfed at one time and insoluble masses of precipitated foreign protein may thus be removed. In the case of pneumococcus antiserums, for example, protective antibody as measured by mouse assay appears to be identical with precipitin and agglutinin. The pneumococcus antibody has been found to enter pneumonic lesions in rats, agglutinating the free pneumococci in the alveoli and thus stopping the spread of the infection.

In the absence of complement, microorganisms are not killed by antibody, and the not uncommon limited therapeutic effect of extremely high titer antibacterial serum has been regarded as an expression of the lack of sufficient complement to take full advantage of the large amounts of antibody present. The bactericidal and lytic activity of complement mediated by antibody probably contributes significantly to effective immunity to at least some kinds of infections.

In some instances antibody may inhibit the reproduction of microorganisms. It was first suggested that antibody might function as an ablastin in pneumococcal infections, with suppression of proteolytic and glycolytic activity, but the evidence in this and other bacterial infections is inconclusive. In general, respiration is not affected by the presence of antibody, and an observed stationary microbial population may occur because the rate of reproduction is balanced by the death rate or because the microorganisms survive but do not multiply.

A reproduction-inhibiting effect of antibody has been demonstrated conclusively only in the case of the protozoan *Trypano*soma lewisi where it is possible to differentiate between lytic activity and inhibition of cell division as attributable to different antibodies. A possibly similar mechanism is operative against the relapsing fever spirochetes (Chap. Thirty-three), but this is not conclusively established. Otherwise there is little evidence that inhibition of reproduction of the microorganism is a significant element in effective immunity.

Essential immunizing antigens. relatively greater importance of antibody to some kinds of toxic substances to effective immunity may be expanded somewhat further. Antimicrobial antibody to various differentiable portions of the antigenic mosaic also vary widely in their contribution to effective immunity. In the case of pneumococcus, antibody to the capsular substance, i.e., type-specific antibody. is primarily concerned in effective immunity to infection, while the contribution of antibody to the somatic antigen is relatively unimportant. In infection with this microorganism phagocytosis is a significant element of the defense mechanism, and the marked association between virulence of the pneumococcus and the presence of a capsule is a consequence of inhibition of phagocytosis by the capsule. Coating of the encapsulated bacteria with antibody facilitates phagocytosis so that antibody to the capsular substance is of primary importance to effective immunity. Or, in the case of streptococcal infection, antibody to the typespecific M antigen, which appears to be very superficially located on the cell, is of greatest importance to effective immunity, while that to the type-specific T antigen and to the group-specific C substance is of lesser importance. In the case of the enteric bacilli and the cholera vibrio, effective immunity is associated with antibody to the heat-stable somatic O antigen and that to the heatlabile H or flagellar antigen is not protective.96

In some instances the antigenic complex that stimulates the formation of protective

antibody may be broken down further. As indicated elsewhere, the typhoid bacillus exists in two forms, the V form which contains Vi antigen, and the W form which does not. Vi-containing strains which lack typhoid bacillus O antigen can be obtained by dissociation, and certain other bacteria, notably a widely used strain of Salmonella ballerup, contain Vi antigen but are unrelated to the typhoid bacillus with regard to the specificity of the O antigen. Immunization with Vi-containing bacteria which lack the typhoid bacillus O antigen, whether typhoid bacilli or not, will protect against infection with V strains of the typhoid bacillus but not against infection with W strains. Conversely, immunization with a W strain of the typhoid bacillus will protect against infection with either V or W forms of this organism, but not against infection with Vicontaining bacteria that lack the O antigen of the infecting strain. It is concluded, therefore, that antibody to the O antigen and that to the Vi antigen are concerned in protection. The components of the other O antigenic complexes occurring in the Salmonella group are not sharply differentiated, in that all seem to contribute to an effective immunity.

The antigens so related to the effective immune response are sometimes spoken of as "essential immunizing antigens." It follows, of course, that not only should an effective immunizing preparation contain the significant antigens, but antigens extraneous to the development of an effective immunity may be omitted. The first consideration has indicated the use of typhoid vaccine prepared from Vi-containing strains, and the second is the basis of the endotoxoid vaccines such as those from the plague bacillus.

Cellular Immunity

The cells of the body have two major roles in immunity to disease, the phagocytosis of microorganisms and other particulate material, and the formation of antibody. The former was established by Metchnikoff at the turn of the century, working first with the water flea, Daphnia, and then with mammalian cells. He differentiated two kinds of

phagocytic cells, the microphages or polymorphonuclear leucocytes, and the macrophages or large mononuclear cells occurring both free and as fixed tissue cells. Although their immediate mobilization accentuates the limited significance of the polymorphonuclear leucocytes, the variety of fixed and free connective tissue cells of mesenchymal

origin, grouped under the general head of macrophage, is of considerably greater importance in resistance to disease.

The distribution and interrelationships of these phagocytic cells may be described

briefly in outline form:6, 90, 91

(A) The predominantly fixed cells of the reticular and loose connective tissue which can be divided into two great groups: (1) fixed and free macrophages. including the reticular cells and (2) the fibroblasts of connective tissue and the endothelial cells lining the ordinary blood vessels.

(1) Macrophage is essentially a physiological designation for almost any large mononuclear connective tissue cell which is predominantly

phagocytic and includes

(a) Fixed cells such as

1. Pericytes (Maximov), fixed, undifferentiated outstretched cells in the adventitia of all the small blood vessels of loose connective tissue throughout the body

2. Reticular cells which, with fibers, form the stroma of all reticular (myeloid and lympathatic) tissues

3. Littoral cells (Siegmund) which line the sinuses of the reticular tissues, liver, hypophysis, and adrenals. In the liver these are designated as Kupffer cells. Although frequently called endothelial cells or cells of the special endothelium, these are in fact either true reticular cells or have greater developmental potencies than ordinary endothelial cells.

These cells can divide by mitosis, become phagocytic, and develop into fibroblasts or practically any other blood or connective tissue cell. Here it is important that they can become phagocytic either in their fixed position (fixed macrophages) or after rounding up and becoming free (free

macrophages).

(b) The free cells occurring in the loose connective tissue are variously known as histiocytes, clasmatocytes, rhagiocrine cells, or wandering resting cells, are either phagocytic or can become so without morphological change, can reproduce by mitosis, and transform into fibroblasts.

(2) The fibroblasts and endothelial cells are morphologically characterized by outstretched, ill-defined cytoplasm, a large oval vesicular nucleus containing dust-like chromatin granules, and small nucleoli.

(a) The fibroblasts divide by mitosis but do not develop into other cells (except in bone and cartilage) and are rarely phagocytic although instrumental in repair

and walling off foreign material. (b) The endothelial cells which line the larger

blood vessels and capillaries (not including the littoral cells) likewise are rarely phagocytic but may be transformed into fibroblasts.

(B) The free connective tissue and blood cells.

(1) The cells of the blood and lymph are classified as to whether of myeloid or lymphoid origin:

(a) The lymphoid cells of the blood include the various sized lymphocytes which, together with monocytes, are termed agranulocytes. They divide mitotically and can develop into macrophages with all the latter's developmental potencies. As they become transformed into macrophages, they show increased amounts of cytoplasm, their nuclei take on macrophage characteristics, and they become phagocytic. These transitional forms are known as polyblasts.

(b) The myeloid cells are the various granulocytes (heterophils or polymorphonuclears, eosinophils, and basophils), the erythrocytes, and platelets. Of these the heterophils are functional in immunity by virtue of their phagocytic activity but are "end" cells which do not reproduce or develop into other cells.

(2) The free mesenchymal cells are lymphoid cells indistinguishable from lymphocytes which occur in varying numbers in reticular and loose connective tissue and here act as precursors or "stem" cells of lymphoid and myeloid cells and hence may give rise to macrophages. They are variously termed lymphocytes, hemocytoblasts, lymphoblasts, myeloblasts, or monoblasts according to varying theories of blood formation. Under normal conditions lymphocytes in lymphatic tissue give rise only to lymphocytes, and hemocytoblasts in bone marrow only to myeloid cells, but under abnormal stimuli they may exhibit their full potencies for development. These stem cells are self-perpetuating, but as noted above, may arise from the fixed mesenchymal cells.

The systems of cells. It will be apparent that the cells primarily associated with defense against invading microorganisms are widely distributed through the body in the blood and lymph, cartilage, bone, reticular (blood-forming) tissue of the myeloid and lymphatic organs, and in the loose connective tissue associated with the skin, omentum, liver, lung, etc. It is customary to group the macrophages into so-called systems of cells. Metchnikoff's macrophage system includes the fixed tissue macrophages and the free macrophages and monocytes of the blood together with the various transitional forms, or polyblasts. Aschoff groups the cells concerned in defense as the reticuloendothelial system (RES) which, in the broad sense, includes the various fixed tissue macrophages and the free macrophages occurring in the inflammatory process. This term is unfortunate in that endothelial cells are not phagocytic, and the reference is to the so-called special endothelium such as littoral cells. The most all-inclusive term is the lymphoid-macrophage system which covers also the lymphoid cells which, as stem cells, may be transformed into macrophages.

to an inflammatory stimulus is characterized by an initial migration of polymorphonuclear leucocytes to the point of injury. These cells are not numerous and soon disappear when the inflammatory material is sterile, but when bacteria are present they continue to migrate from the blood vessels and actively phagocytose the invading microorganisms. The functions of this first line of defense, although important, are strictly limited, since the cells are short-lived and do not multiply *in situ* but must be continuously recruited from the blood stream.

More important to local defense are the lymphoid cells of the blood and lymph, the lymphocytes and monocytes, which also migrate from the blood vessels but which, unlike the polymorphonuclear leucocytes, are long-lived and multiply in the tissues. These stem cells may be transformed to macrophages which, together with macrophages already present in the area, actively phagocytose and digest the invading microorganisms. When large bodies of foreign material are present, the macrophages may fuse to form foreign body giant cells; when microorganisms are indigestible they may form giant cells around them such as the epithelioid cells of the tubercle. Their progressive development into fibroblasts supplies the active elements for regeneration and repair, the formation of scar tissue and the walling off of foreign bodies.

Certain other cells may take part in the late stages of the inflammatory process; the eosinophils, for example, may play a part in the detoxification of proteins and their disintegration products.

The inflammatory reaction 40. 55. 80. 92 plays an important part in eliciting this cellular response and contributes to the tissue injury produced. A polypeptide, designated leucotaxine, which is found in exudate fluid, increases capillary permeability and provides a chemotatic stimulus for the early heterophil migration; its activity is antagonized to some extent by substances such as cortisone and hydrocortisone. A leucocytosis-promoting factor of pseudoglobulin

nature, found in the α -globulin fraction of exudate, is also produced in the inflammatory process that is associated with hyperplasia of granulocyte precursors in the bone marrow. A leucopenic factor, of polypeptide nature, is formed also, together with a toxic euglobulin called necrosin. The latter is absorbed from the inflammatory lesion, possibly producing injury elsewhere, especially in the liver and kidneys. This last is an example of a toxic substance of host rather than microbial origin as described elsewhere (Chap. Nine).

General defense. When a stimulant is distributed over a large part of the body the reaction is regarded as a general rather than local one; such distribution, however, usually means that the stimulant is in the blood stream and is combated by the cells of organs most closely associated with the blood, i.e., the spleen, liver, and bone marrow, and in some cases general reactions may be regarded as local ones in strategically placed organs.16 The same types of cells are involved as in the local reaction. the polymorphonuclear leucocytes being mobilized first, with the macrophages playing the more important role. Endothelial cells, although in contact with bloodborne material, show very little phagocytic activity, and the fibroblasts are rarely active.

The cellular response in immunity. 23, 45 The above reactions, which are observed to occur in the nonimmune animal, are markedly accentuated in the immune animal, and there is an increase in the number of macrophages frequently designated as a hyperplasia of the reticuloendothelial system. The cooperative role of the humoral antibodies is of considerable significance: 102 an antigen is localized by agglutination if cellular or by precipitation if in solution, and in either case the material is made more readily phagocytable by opsonization. Bacteria injected into the blood stream, for example, are rapidly removed in the immune animal by the fixed macrophages of the liver and spleen,73 and staphylococci injected into the skin are localized and phagocytosed in great numbers. When antigen and antibody meet in the tissues of an immune animal, there are not only localization and opsonization of the antigen, but also a much heightened inflammatory reaction including a speeding up of the cellular response.

SITES OF ANTIBODY FORMATION^{54, 63, 87}

Although antibody is ordinarily titrated and studied as it occurs in serum, serum antibody represents that synthesized by cells and spilled over into blood and body fluids. The function of cells in the synthesis of immune globulin is inferred from several lines of evidence, including interference with antibody formation by inhibition, removal or destruction of certain tissues, antibody production by transplanted cells, the distribution of antibody activity within the tissues, and the cytological events coinciding with antibody formation. While it is believed by some that both normal and immune globulin are synthesized in the same way, antibody resulting from a deviation in the normal synthetic processes, it does not necessarily follow that conclusions regarding the synthesis of immune globulin are completely applicable to the processes of synthesis of normal serum protein.

The relation of cells of the macrophage system to antibody formation is indicated by the results of interfering with their function. Blockade, by the inoculation of relatively large amounts of particulate material, such as carbon particles, so that all the phagocytic cells are packed with the substance and phagocytosis is sharply inhibited, markedly reduces the immune response. Similarly, removal of tissue rich in the elements of the macrophage system, as by splenectomy, also markedly modifies the the formation of antibody.

Gross injury to the cells of myeloid, as well as lymphoid, origin by ionizing radiation also affects antibody formation. When administered prior to, or within a short time after, the inoculation of antigen, irradiation sharply reduces or even eliminates the immune response, although a pre-existing immune response is not affected. The effect of lethal, or near-lethal, whole body x-irradiation is so marked that the homograft reaction disappears. Lethal doses of ionizing radiation destroy the bone marrow, and substantial protection is conferred by inoculation of the treated animal with bone marrow from normal animals. The irradiated animal does not give a homograft reaction to the transplanted cells, and, although they are eventually replaced when the animal survives, they persist and multiply with the appearance of blood cells of myeloid origin characteristic of the donor to give a radiation chimera. Irradiated animals similarly do not react to skin, tumor, and other tissue grafts.

Antibody formation in vitro.93 If cells are primarily responsible for antibody formation, it should follow that such cells would form antibody in tissue culture. It is well established that explants of lymphoid tissue from immunized animals,82 i.e., the cultured cells, are already committed to the immune response.83 The induction of antibody formation in similar cultures of cells from normal animals was reported as early as 1912, but for many years the results could not be repeated. More recently, a number of reports of the induction of the antibody response by normal cells in culture in response to antigenic stimulus have appeared. 11, 32, 58 It is not altogether clear what conditions are required. Positive results have been obtained by the inclusion of RNA, apparently of the mRNA type, from immunized animals in the cell culture,14,28,52 but this would appear to be a case of adoptive immunity (see below). The initiation of antibody synthesis in vitro in paired explants of spleen and thymus⁷⁶ is of interest in connection with the apparent significance of the thymus to the immune response (see below).

Cell-mediated antibody formation.¹³ Explants of lymphoid tissue from immunized animals will also form antibody in vivo, i.e., when inoculated into a normal animal.⁸⁶ This occurs even when the recipient, such as the immature or irradiated animal, is unable to form antibody, or as in the case of the appearance of antibody to the typhoid bacillus in an individual having agammaglobulinemia but receiving lymphoid tissue from an individual immunized with typhoid vaccine.

Further, hypersensitivity of the delayed type (see below), which is not passively transferable by serum, may be transferred by the inoculation of a normal animal with lymphoid tissue from a sensitized animal. The occurrence of an immune response mediated by cells adds a third facet to acquired immunity which has been called "adoptive" immunity.^{5, 17, 59}

Antibody-forming cells. The association of antibody formation with lymphoid tissue is of long standing, and there is an impressive amount of evidence in the immune

response of the intact animal supporting it. For example, a number of workers, including Erich and his co-workers and Harris and his co-workers, have carried out the kind of experiment in which minimal amounts of antigen are inoculated into the footpad of an experimental animal, usually the rabbit, and the afferent and efferent lymph from the regional lymph nodes examined for antibody activity. This kind of experiment is subject to control by giving different antigens on either side of the animal and titrating the lymph specimens for both kinds of antibody activity. In such experiments much more antibody to the locally inoculated antigen is found in the lymph draining from the node, the greater part of which is found contained in the lymphocytes. Such evidence is clearly indicative of the ability of lymphoid cells, but not necessarily mature lymphocytes, to form antibody.

In the same vein, the role of various organs in antibody formation may be examined. This is necessarily indirect because antibody-forming tissues apparently do not retain antibody. The importance of the spleen, for example, is indicated by the

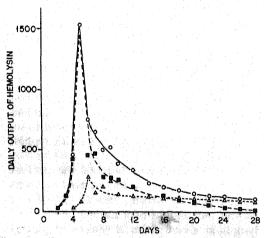


Figure 79. The mean daily output (not serum titer) of hemolytic antibody to the sheep erythrocyte by the rabbit following a single intravenous inoculation of antigen. The total output by the intact animal (circles), the nonsplenic output (triangles) by splenectomized animals, and the splenic output (squares) by difference are shown. Note the high peak and precipitous decline in splenic antibody output and the lower peak but continued production of nonsplenic antibody. (Taliaferro.)

relatively large amounts of antibody formed in tissue culture by explants from immunized animals, but the red pulp is more active than the follicular tissue.

The cytological evidence for the formation of antibody by cells is the localization of antigen and antibody within cells, and the cell types and changes associated with antibody formation.24 In the first instance, labeled antigens, viz., coupled dye-proteins and antigens containing I¹²⁷ or I¹³¹, or S³⁵, may be used, or antigen may be located by treatment of the cells with fluorescent antibody. Similarly, antibody may be located within cells by treatment with fluorescent antigen. Studies of this kind have shown that antigen is found in phagocytic cells of the macrophage system, but that antibody is found, not in the same cells as antigen, but in plasma cells or immature plasma cells whose active nucleic acid synthesis is shown by their affinity for methyl green-pyronine. Such cells also appear in cell cultures of explants from immune animals on secondary antigenic stimulus in culture.72

In a general way, the immune response, especially the secondary or anamnestic response, is characterized by hyperplasia of the lymphoid-macrophage system. More precise studies have shown that the antibody response is associated with the occurrence of the large pyroninophilic cells found to contain antibody. For example, in their studies on the rabbit lymph node, Leduc, Coons, and Connolly⁴⁸ found relatively few of these cells in the normal animal, but on antigenic stimulation larger numbers appeared in the medullary portion of the node, multiplied and differentiated, and accumulated increasing quantities of antibody in the cytoplasm until the development of the cells reached a point at which they appeared to be mature plasma cells. The primary and secondary immune responses were similar, but many more antibodycontaining cells were found in the anamnestic reaction. A similar cellular response, but without identification of intracellular antibody, was observed in the spleen explants noted above, the red pulp explants containing large numbers of similar cells, described as "transitional," and the follicular tissue explants very few.41

Antibody formation by single cells. 61 It has been found that single lymphoid cells,

taken from an animal immunized with more than one antigen, form antibody to one or another of the immunizing antigens; i.e., individual cells form antibody of a single specificity and the observed multiple antibody response is apparently attributable to the pooling of the antibody output of many cells. This is apparently not invariably true. for the formation of antibodies of different specificities by single cells has been described,2 but the proportion of such cells appears to be very small.62 Formation of antibody by single cells may be observed as an agglutination of the bacterial antigen by antibody secreted by a single cell in an aqueous droplet in paraffin oil, and has been demonstrated also in frozen sections of lymphoid tissue from immunized animals by the fluorescent antibody technique. These observations have been taken to confirm the clonal selection theory of antibody formation (Chap. Thirteen), but they are consistent with, rather than confirmatory of, this theory.60

Antibody response in vivo. A general pattern in the relation of cells and tissues to the formation of antibody emerges from considerations such as the foregoing. The presence of antigen, or some fragment of antigen, within the antibody-forming cell

is presumably required to provide both stimulus and template. 12 Under experimental conditions demonstrable antigen may persist for extended periods, as, for example, the persistence of Vi antigen in the mouse for 231 days, an appreciable portion of the life span of this animal.29 It seems not to be necessary, as once thought, that such cells be phagocytic. Apparently the first step in the sequence of events known collectively as the immune response is phagocytosis of the antigen which is then transferred, possibly as a fragment, to lymphoid cells. Such transfer of antigen between macrophages and lymphoid cells has been observed to occur by cytoplasmic interaction,77 which apparently increases under antigenic stimulus. The presence of antigen in lymphocytes has been directly demonstrated by electron micrography and radioautographic methods.39 This initial step is that which is interfered with in blockade and similar procedures, and the preparatory stage accounts, in part at least, for the induction period elapsing between inoculation with antigen and the first appearance of antibody, and is a radiosensitive stage in the immune response. Some breakdown of the antigen molecule is possibly the basis of the apparent requirement that the antigen be me-

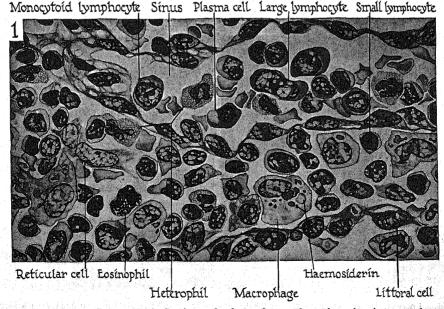


Figure 80. Cells of macrophage system. Sections of spleen of normal monkey showing a portion of a venous sinus and a Billroth cord in the red pulp; note the typical structure of the nongranular leucocytes, the reticular cells with indeterminate cytoplasm, and the rounded macrophages within the cord. \times 1240. (Taliaferro and Mulligan.)

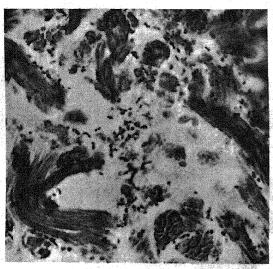
tabolized to some degree at least by the host tissues, and the rapid synthesis of antibody coincides with the disappearance of antigen in the blood.

The relative contribution of the antibodyforming mechanisms in various tissues to the total immune response is dependent upon the amount of antigen given and the route of its inoculation.78 When only small amounts of antigen are given other than intravenously, antibody tends to be formed locally, as in lymphoid tissue draining the area of inoculation, but large amounts of antigen given similarly spill out of the local area to stimulate antibody formation more generally. When the antigen is inoculated intravenously, the spleen is the site of early, and most intense, antibody formation, while antibody formation in other tissues tends to be somewhat less rapid but persists after the splenic response has declined.19 There has been some interest in mass immunization by inhalation of antigen, e.g., aerosol immunization, which tends to approximate in effect the intravenous route.21,30 While, then, the spleen is the most important source of antibody on appropriate initial antigenic stimulus, and as a consequence the antibody response is markedly affected by splenectomy, in the hyperimmunized animal the contribution of the spleen becomes less important, and antibody of nonsplenic origin becomes predominant.

The oral route, for many years somewhat discredited, has become the subject of renewed interest. It produces a superior coproantibody response as a consequence of local antibody formation in the lamina propria, but antigen by this route, other than infection produced by attentuated microorganisms as in live poliovirus vaccine, is inefficient as judged by serum antibody response.

Local immunity. The defense mechanisms functioning in the immune state have been assumed to be general ones and, for all practical purposes, equally effective throughout the body. It has been suggested, however, that these mechanisms are localized, or obviously accentuated, in certain tissues, not necessarily in tissues containing a large proportion of cells of the macrophage system, but tissues such as the skin, the intestinal mucosa, and the nasal mucosa.

Although attributable in some degree to the nature of the local defense factors, such as pH and the like, the predilection of an invading microorganism for some particular part of the body such as the central nervous system might be regarded as indicative of a relative susceptibility of certain tissues and, conversely, a relative resistance on the part of others—in Ehrlich's terminology, the presence or absence of cell receptors. In a disease such as typhoid fever or erysipelas, for example, it should be necessary then that only the intestinal mucosa or the



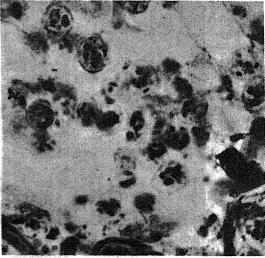


Figure 81. The cellular response in the immune animal. Rabbits injected subcutaneously with virulent pneumococci. Left, normal animal showing a typical spreading lesion with many extracellular pneumococci and minimal phagocytosis by polymorphonuclear leucocytes, Right, immune animal showing marked phagocytosis with intracellular agglutination and few extracellular bacteria. Sections stained by Gram's method; × 1050, (Cannon.)

skin be immune, the other tissues being already resistant. The evidence upon which it has been based is generally regarded as relatively weak, and local immunity in this sense probably does not exist.

Muco-antibody.68 There is. however. good reason to think that local immunity, not of a particular tissue such as the skin, no matter where it may be in the body, but of a local area, does exist, and is associated with local concentrations of antibody. Such antibody usually occurs admixed with mucus at mucous surfaces, and in this and other secretions is usually IgA. That present in respiratory mucus reflects the rise and fall of serum antibody titer, and is considered to represent extravasated serum antibody. In other areas, notably the bowel and the vagina, such antibody titer does not parallel serum antibody titer, but reaches a peak earlier and subsides while serum antibody continues to rise. Local formation of antibody, indicated by a superior local response and markedly inferior serum response following local application of antigen, is consistent with this discrepancy; *i.e.*, the later peak in serum titer is a consequence of the cumulative nature of serum titer, while the local titer is a reflection of the rate of antibody formation.

Such antibody is called muco-antibody because of its occurrence at mucous surfaces, and in the case of that found in the bowel, coproantibody. A variety of antibodies have been described as occurring locally, including those to dysentery bacilli and cholera vibrios, to poliovirus 51,53 and to the α -toxin of Cl. welchii in the bowel, to Trichomonas in the vagina, and to leptospira and poliovirus in the urine. 49,70

Natural Immunity

As pointed out elsewhere, the defenses of the normal animal against infection are of two general kinds. The first, characterized by nonspecificity with respect to the infectious agent, has been called resistance and has been discussed earlier. The second kind showing a greater or lesser degree of specificity, is considered here as natural immunity.

It is the rule rather than the exception that pathogenic microorganisms are sufficiently closely adapted parasites that they are able to establish infection only in more or less closely related species of hosts. Thus it is perhaps not to be expected that the same parasite will be able to infect organisms as widely different as higher plants and mammals. It is of particular interest, therefore, that *Pseudomonas aeruginosa*, a not uncommon pathogen of man and higher animals, has been found able to infect tobacco plants and may be identical with the plant pathogen *Phytomonas polycolor*.

While in Ehrlich's terminology it might be said that the cellular organization of the plant lacks receptors for the human pathogen, there is evidence of a positive type of resistance to such infection. Antibiotic substances, similar to those produced by bacteria and fungi, have been found in a number of higher plants. Thus, extracts of cabbage, turnip, onion, and barberry plants inhibit the growth of coliform bacilli and Bacillus subtilis; chlorophyll and related compounds have been found to inhibit the growth of the tubercle bacillus; and substances which inhibit the growth of Staphylococcus aureus and coliform bacilli have been found in a large number of angiosperms. None of the plants, however, is known to produce anything resembling antibodies.

Specific immunity. Natural immunity is often closely associated with the specific immune response, an immunity which is expressed in the form of the bactericidal and opsonic powers of normal blood, in the presence of the so-called normal agglutinins and the like. Such antibodies are generally present only in low titer, and the immunity associated with their presence in man is of a low grade but is possibly a significant part of that complex designated resistance. In many instances antibodies may be found under circumstances in which it is highly improbable that the animal has come in contact with the infectious agent; it has been noted, for example, that cattle serums will not infrequently neutralize the yellow fever virus and, more often, contain agglutinins

for bacteria such as the typhoid and dysentery bacilli, the cholera vibrio, and certain of the rare varieties of Salmonella. Although present in low titer, such normal agglutinins appear to be no different from the immune agglutinins in adsorbability, heat resistance, and the like, and such antibody activity occurs as all three classes, IgG, IgA, and IgM, of immunoglobulin. 15 The normal macrophage response does not involve this specific element, for all kinds of intact particles are phagocytosed. The question of the origin of these antibodies is, then, of some significance, and it may be asked whether or not specific antibodies may be formed in the absence of antigenic stimulation.

Natural antibodies.8 That the formation of some antibodies is genetically determined and takes place in the absence of antigenic stimulus is established in the case of the antibodies which determine the human blood groups. It is not unreasonable to suppose that in other instances the arrangement of polar forces on the surface of the normal globulin molecule might be such as to make possible to a greater or lesser extent the specific adsorption of antigenic substances. In fact, if the clonal selection theory of antibody formation (Chap. Thirteen) is valid, serum globulin would contain antibody, at least in trace amounts, to all possible antigenic specificities. The probability that this has any such practical reflection, if indeed it does occur, is not great, and it is unlikely that such an explanation is generally valid.

Inapparent infection. A second possibility that accounts for the presence of normal antibodies is that of inapparent infection. There is no reason to doubt that the average individual is exposed more or less continuously to a wide variety of microbial antigens, beginning post partum and extending throughout life. The microorganisms constantly present in the form of normal flora, especially of the respiratory and gastrointestinal tracts, provide a source of antigens seeping into the tissues. The protective activity of pooled normal adult gamma globulin against a wide variety of infectious agents⁵⁷ is no doubt an expression of such antibody content of normal human serum.

A case in point is that of the appearance of diphtheria antitoxin in the blood in the absence of clinically recognizable infection;

it is well known that the proportion of Schick-negative individuals increases in succeeding age groups. It is definitely established that the indicated formation of antitoxin is an immunity resulting from inapparent infection, i.e., the carrying of virulent diphtheria bacilli in the throat, and the antigenic stimulus of the presence of small amounts of diphtheria toxin. The agglutinins for Flexner dysentery bacilli and some of the common species of Salmonella associated with food poisoning, not infrequently present in normal human serums to titers of 1:80 to 1:160, in all probability are a consequence of subclinical infections. It is probable that such a sequence of events occurs in a number of diseases and that socalled normal antibodies, such as the neutralizing power of normal adult serum for poliomyelitis virus, measles virus, and other infectious agents, are, in fact, immune antibodies. The manner in which a pseudoracial immunity may be produced through infection has been discussed elsewhere.

Common antigens. As indicated above, however, there are many instances in which it is extremely unlikely that the animal whose serum agglutinates a given bacterium came in contact with it either as a mild infection or otherwise. It is difficult to conceive, for example, of the ordinary cow in the United States coming in contact with the cholera vibrio. In such cases it is not necessary to assume either that the observed antibodies arose de novo through some genetic or maturation mechanism or that there was contact with the particular microorganism. It is only necessary to assume that the animal has come in contact with an immunologically similar antigen. In this case the cholera vibrio is immunologically related to Brucella, and brucellosis, in cattle or in man, results in the appearance of cholera agglutinins in the serum.22 The likelihood that this will occur is clearly a function of the frequency with which immunological relationship or identity is shared by diverse organisms and the probability of contact with the unrelated form.

In addition to heterophile antigen, whose apparent random occurrence has been discussed elsewhere, immunologically similar antigens have been found to occur in a variety of seemingly unrelated organisms, and it is probable that many more have not as

vet been discovered. For example, crossreactions occur between type 2 pneumococcus polysaccharide and type B Friedländer's bacillus, certain species of yeasts, gum acacia and other vegetable gums, and some strains of coliform bacilli; the polysaccharide of type 14 pneumococcus is immunologically similar to the specific antigen of human blood group A; a constituent present in peptone is immunologically related to certain streptococcus antigens (group C); the capsule of the anthrax bacillus appears to be immunologically identical with that of B. mesentericus; the plague bacillus and paratyphoid B are immunologically related. From these examples it will be clear that immunologically similar antigens may be distributed in an apparently random fashion in unrelated organisms, and it is probable that in some, if not many, instances in which antibodies may be demonstrated for some microorganism with which infection or contact is unlikely, the antibodies are immune rather than "normal" and a consequence of exposure to a similar antigen.

In keeping with this discussion it may be pointed out that there is a strong possibility that there is no such thing as normal specific antibacterial immunity and that immunity in which a specific antibody may be demonstrated is one that arises as a consequence of exposure to the antigen. The incubation period following injection, for example, provides the opportunity for an immunological response that is difficult to rule out in many instances.

Acquired Immunity

Acquired immunity, as contrasted with natural immunity, is invariably an expression of a response to an antigenic substance. It is of two general kinds (in addition to the experimentally induced adoptive immunity described above), *viz.*, active immunity in which the immune individual forms his own antibody in response to an antigenic stimulus, and passive immunity in which the individual acquires preformed antibody produced by some other individual.

ACTIVE IMMUNITY

Active immunity is acquired as a consequence of exposure to the microbial antigen, either inadvertently as in infectious disease or inapparent infection as described above, or by deliberate inoculation with an appropriate antigenic preparation to give artificial immunization.⁶⁶ The last is, as pointed out by Edsall,²⁰ an attempt to get something for nothing. The objective is an effective immunity to disease, not a simple immune response in the technical sense, and it is in this connection that the immunogenic potency of antigens is assayed by the active protection test (Chap. Fourteen).

A number of immunizing preparations

and methods of producing an active acquired immunity may be used. These are:

- (1) The inoculation of living, fully virulent bacteria
 - (a) Rarely by a route favorable to infection
 - (b) Occasionally by a route unfavorable to infection
 - (c) In conjunction with protective antiserum
 - (d) Which are drug-dependent
- (2) The inoculation of attenuated bacteria of greatly reduced virulence for the host
- (3) The inoculation of bacteria killed by
 - (a) Heat
 - (b) Antiseptics
- (4) The inoculation of bacterial products
 - (a) Secreted during life
 - (b) Extracted from dead cells
- (5) The inoculation of bacteria unrelated to the production of the specific infection

These may be briefly illustrated.

(1) Immunity produced by the introduction of living, virulent microorganisms is practically identical with the immunity that results from an attack of disease after natural exposure. In experimental work the varying facility with which this mode of immunization can be effected is in part dependent upon the susceptibility of the organism to the particular parasite, and a highly susceptible animal can be immunized in this way only with great difficulty or not at all. The successful use of living cultures

involves the administration of small nonfatal doses which are increased as immunity develops.

The relative insusceptibility to infection by some particular route may be taken advantage of, as in Ferran's method, now superseded, for protective vaccination against Asiatic cholera; natural infection occurs via the alimentary tract and subcutaneous injection of the virulent vibrios is followed by a local inflammation but not by a general infection with serious consequences.

The simultaneous administration of virulent microorganisms and protective antiserum provides preformed antibody for combating the invader while allowing the immune reaction of the host to develop. This method is seldom used for the immunization of human beings but is common in some other instances; hogs may be immunized against hog cholera, for example, by simultaneous injection of virus-containing blood and antiserum. As has been described elsewhere (Chap. Seven), strains of pathogenic bacteria may be made drug-dependent in that they cannot grow except in the presence of the antimicrobial agent, and are virulent only when the host is treated with the drug. When such strains are inoculated into susceptible animals, they are unable to produce infection and disease.

(2) As indicated elsewhere, the virulence of pathogenic microorganisms may be greatly diminished in a variety of ways. such as cultivation under unfavorable environmental conditions and by passage through animal species other than that of the host in question.26 Perhaps the most familiar examples of the use of such attenuated material are those of smallpox vaccination and inoculation against rabies. In the first instance the virus of cowpox protects against smallpox infection, and in the second, rabies virus from dogs is "fixed." in Pasteur's terminology, or attenuated by serial passage in rabbits. This method is particularly useful in immunization against the virus diseases.

(3) Suspensions in physiological salt solution of bacteria killed by heating to 55° to 60° C. for 30 minutes or by treatment with formaldehyde or phenol are widely and successfully used in man against typhoid and paratyphoid fevers and against a variety

of microorganisms in the laboratory. Such vaccines are, in general, most useful in infections in which the microorganism does not produce a soluble toxin but contains an endotoxin. This method has the obvious advantage of avoiding all danger of infection while at the same time introducing into the body the substances most intimately connected with the bacterial cell and its activities.

(4) The use of products of the bacterial cell for immunization purposes finds widest application in the case of those organisms which produce soluble toxins. As pointed out above, a solid immunity against diphtheria may be secured by stimulating the production of antitoxin. It is possible to build up such an immunity by using extremely small amounts of unmodified toxin in the early injections with gradual increases as immunity develops. In general, however, it is much more satisfactory to use neutralized or detoxified toxin. In the first instance a neutral mixture of diphtheria toxin and antitoxin (TAT) is frequently used with satisfactory results; the complex breaks down slowly in the body, liberating free toxin which acts as the antigen. Toxoid prepared by treatment with formalin which has lost its toxicity but retains its antigenicity is generally used at the present time in a partially purified form precipitated by alum. The alum-precipitated toxoid is of some advantage since the toxin-alum complex breaks down slowly in the tissues, liberating toxoid slowly to provide a prolonged antigenic stimulus.

As pointed out earlier, antibodies to certain portions of the antigenic mosaic of the bacterial cell contribute more to effective immunity than antibodies to other portions. Untoward reactions resulting from inoculation with the intact cell may be due in part to antigens unimportant to the development of effective immunity, and if these are eliminated from the immunizing preparation, the amounts of essential antigen may be increased. Considerations such as these have led to chemical and immunological fractionation of plague bacilli, for example, in efforts to prepare more effective immunizing antigens.

(5) Some degree of immunity toward specific infections may be developed by the use of certain kinds of bacteria or bacterial

401

products entirely foreign to the infection in question. This protective effect seems to be of two general kinds. In the one, when the apparently unrelated microorganism contains antigens in common with the pathogenic form, the protection conferred is that of the conventional antibody response. The other is transitory and a manifestation of the protective effect of endotoxins of the gram-negative bacilli against some kinds of microorganisms. pathogenic This nomenon is well established and is, to a considerable degree, a consequence of a stimulation of the cellular defense mechanisms. 7, 44, 74

Irrespective of the antigen employed, the animal body requires time to respond with antibody production, and for a high degree of immunity successive antigenic stimuli are required. Early injections may consist of killed bacteria, followed by the inoculation of living, attenuated, or virulent cells. The process of forcing a very high degree of immunity upon an experimental animal is often termed hyperimmunization. The function of adjuvants in providing a persisting depot of antigen, and therefore a persisting antigenic stimulus to replace repeated inoculations, has been discussed earlier.

The immune response. The immunity produced is variable to some degree and dependent upon the efficacy of the antigen used. Some kinds of bacteria are good antigens, such as the typhoid bacillus, while others, such as the gonococcus, are poor antigens, and no effective immunity may be produced. The reason for this is not known. When an active immunity is produced, however, it is generally of long duration and effective over a period of years.

The immune response, as indicated by antibody titer, generally is apparent by the second of a series of injections, and the titer increases with succeeding injections until an upper limit is reached. With cessation of inoculations the antibody titer slowly declines and within a few weeks has reached a very low level. Subsequent injection brings about an immediate antibody response, much more rapid and pronounced than that of the initial immunization.

Ontogeny.^{34, 79} It has long been known that animals in the neonatal period are relatively poor antibody producers, and the phenomenon of immunological tolerance

SAUMA, BAALO LIBANA

(Chap. Thirteen) rests upon the basis that the highly immature cells of the fetus are unable to recognize the foreign nature of antigenic substances. Consistent with this, little or no γ -globulin is synthesized by the fetus, and in man and certain other mammals the γ -globulin, and antibody, content of fetal serum is of maternal origin (see below). In the neonatal human infant there is a steady decline in serum γ -globulin, to the extent that it may even reach a transient agammaglobulinemic level, but production is initiated in three to 12 weeks, although adult levels may not be reached for six, or even 10, months.

Failure to respond to antigenic stimulus with antibody production is attributable in large part to incomplete development of antibody-forming mechanisms, but there is evidence also that the presence of antibody of maternal origin has a depressive effect. However this may be, the potential antibody response of the fetus and the newborn has generally been underestimated, and, particularly in the latter, an antibody response may be elicited to strong antigenic stimulus, though it is usually inferior to that obtainable after further development. The antibody produced is almost entirely IgM.

The point in development at which a reasonable degree of immunological competence is reached, i.e., at which an antibody response is demonstrable, varies among species and with the kind of antigen. In the fetal lamb, for example, antibody to bacteriophage antigen is produced after 35 to 40 days of gestation, the homograft reaction is apparent at 85 days, but diphtheria antitoxin is not formed at six weeks after birth. Immunologic competence appears to develop much more rapidly in marsupials such as the opossum, in which antibody production is possible as early as 11 days after birth, but considerably later in pigs, cattle, guinea pigs, and rabbits. The human infant shows a detectable immune response to antigens such as diphtheria toxoid, poliovirus, and enteropathogenic coliforms at perhaps one month post partum, but the response is inferior to that obtained later. It is not possible to equate such antibody response entirely in terms of potential immunological competence, for contact with antigen appears to stimulate development of the antibody-forming mechanisms.

STEEN BUT

Lymphocytopoiesis. Immunological sponsiveness appears to parallel closely lymphocytopoiesis,* and the role of the thymus in mammals has been of particular interest.⁵⁶ In man, and many other animals, the thymus appears to be essentially the sole source of antenatal lymphocytes which arise by transformation of epithelial cells, beginning at about two months in man, and at varying times in other species. Thymic lymphocytes migrate peripherally to establish centers for the development of lymphoid structures and, with advancing age, contribute in large part to the lymphocyte population. Proliferation of lymphocytes in lymphoid structures, such as the spleen and lymph nodes, is normally limited, but is markedly accentuated by antigenic stimulus. In the extreme case of germ-free animals, the lymph nodes are atrophic, with little or no evidence of mitosis, while the thymus is fully developed and actively lymphocytopoietic. On antigenic stimulation germinal centers appear in the spleen, lymph nodes, and other lymphoid tissues, showing mitotic activity and active lymphocytopoiesis coincident with the production of antibody. Such peripheral centers, then, appear to constitute the morphologic facet of the antibody-forming mechanism, although the thymus continues to manufacture lymphocytes at a steady rate.

Thus in the mouse, thymectomy within 24 hours of birth initiates a wasting syndrome characterized by loss of weight, cachexia, etc., which is fatal in perhaps a month. Such animals are immunologically unresponsive, and the terminal lymphocytopenia is marked. When the removal of the thymus is delayed, this syndrome is not produced and the animals are immunologically responsive. The persisting significance of continued lymphocytopoiesis in the thymus is indicated by failure of animals subjected to delayed thymectomy to recover from radiation damage; i.e., following radiation destruction of peripheral lymphoid tissue, regeneration would appear to be dependent upon thymic lymphocytes. There is evidence also that the role of the thymus. at least in mice, may also include a humoral factor affecting lymphocytopoiesis. 47

While the thymus plays a primary part in

lymphocytopoiesis and the development of the antibody-forming mechanisms in many animals, in certain species other lymphoid tissue may take over this function in whole or in part. It is firmly established that the bursa of Fabricus in the chicken is a highly significant element in immunological responsiveness, ⁹⁷ so much so that it has been referred to as a "cloacal thymus." There is evidence too that the appendix in the rabbit functions similarly.⁸⁵

Polyvalent and multiple antigens. high specificity of the immune response may require more than one strain or kind of microorganism to give effective immunity to a disease entity. Influenza, for example, is caused by a number of antigenically distinct strains of the virus with little or no cross-protection among them, and an immunizing preparation producing an immunity to the disease must include the relevant strains. Similarly, cholera vaccine often contains the two major serotypes of the vibrio, and typhoid vaccine may be combined with the paratyphoid A and paratyphoid B bacillus vaccines. Such immunizing preparations are known as polyvalent vaccines. The use of polyvalent vaccines is limited in practice by the number of components which are required. The total amount of antigenic material that may be administered without unduly severe reactions is limited. When, then, many components are required, the amount of each consistent with a tolerable reaction to the preparation may be so limited that no individual component is sufficient to provoke an effective immune response.

For the purely practical purpose of limiting the number of parenteral inoculations, it may be desirable to combine widely different immunizing agents as a multiple antigen. Commonly used combinations are, for example, pertussis vaccine and tetanus and diphtheria toxoids in some combination. The use of multiple antigens, and polyvalent vaccines, raises the question of whether or not the independent immune response to each of the antigenic components is adversely affected by the presence of the others, i.e., whether the individual antibody responses are suppressed when they occur simultaneously. In some combinations there is some suppression of the immune response, but in others there may be an accentuation. The latter effect appears

^{*}The term lymphocyte is used loosely here to cover the range of cells, including plasma cells, of the lymphoid series.

often to be due to an irritant effect of one antigenic preparation that accentuates the response to an accompanying relatively bland antigen. Various combinations of antigens behave differently, and the immunizing efficiency of multiple antigens cannot be generalized. 10, 35, 67

Precocious protection. 10, 35, 67 It has been observed from time to time that within a few hours after inoculation with an immunizing antigen, the recipient develops an appreciable resistance to challenge inoculation which is usually, but not invariably, specific. For example, such a transient resistance develops rapidly after inoculation with typhoid or pertussis vaccine, in the latter instance reaching a peak in eight to 10 days and prior to the appearance of demonstrable antibody. The phenomenon has also been observed with tetanus and diphtheria toxoids and with typhus fever rickettsiae.

This transitory protective effect is distinct from the active immune response and is usually regarded as an interference effect, based upon competition between the inactivated immunizing antigen and the challenge inoculum for tissue receptors. As such, it is closely similar to, if not identical with, the interference phenomenon among viruses (Chap. Four).

PASSIVE IMMUNITY

In contrast to active immunity, passive immunity involves no active generation of protective substances by the immunized animal. The latter is simply the recipient of antibodies formed in the body of another animal and transferred to the individual to be protected.³⁴ Such passive transfer occurs in nature from mother to offspring, *in utero* via the placental circulation in man, apes, and rodents, in which there are only two layers of cells between the maternal and fetal circulatory systems, and after birth via the colostrum in ruminants, in which there are four layers of cells between the two circulatory systems.

The nature of the transfer of maternal antibody to the fetal circulation is not altogether clear. It is probable that the placenta does not play a significant part in antibody formation, and presumably it is a process of diffusion from the maternal

circulation. It has been observed that antibodies to different antigens are transferred to varying degrees; viz., that to mumps and Japanese B encephalitis is relatively complete, while antibody to vaccina and poliovirus shows considerable disparity between maternal and fetal titers.⁵⁰ Of the antibody classes, only IgG appears to be transferred in utero.

Absorption of antibody present in colostrum and milk occurs readily, at least in the immature animal, 9, 37, 38 and makes possible passive immunization of the newborn when transfer of antibody from the maternal circulation is not effective. Local formation of antibody occurs in the lactating mammary gland, and it appears to respond exceedingly rapidly to local antigenic stimulus. There is some evidence suggesting that local antigenic stimulus may be provided by microorganisms present in the suckling young, with consequent rapid return of antibody to the infant whose antibody-forming capacity is as yet poorly developed.

Artificial passive immunization is brought about by the injection of immune serums such as the antitoxic serums of horses immunized against diphtheria toxin, tetanus toxin, and similar antigens, or of convalescent serums taken from humans who have recovered from the disease in question. As noted above, the γ -globulin separated from pooled adult plasma contains antibodies to the infectious agents encountered early in life, and is used for passive immunization of the young against infections such as measles. Passive immunization is most effective with antitoxic serums; with exceptions such as virus-neutralizing serums, antipneumococcus serums and the like. antibacterial serums are generally not highly effective either prophylactically or therapeutically.

Unlike active immunity, passive immunity is not of long duration, generally not more than two or three weeks. Repeated injections of tetanus antitoxin, for example, must be made at weekly intervals as long as the danger of infection remains. Horse serum globulin acts as a foreign protein in the human body and tends to be eliminated as such. Passive immunization with homologous serum is possibly of somewhat longer duration; diphtheria antitoxin passively transferred in utero has been found to have a half-life of four and one-half weeks.

Hypersensitivity

The response of the animal body to the presence of antigen in the tissues is clearly an advantage when the antigen is a toxin or the cell substance of a pathogenic microorganism. It was early apparent, however, that initial inoculation of an antigen may so sensitize the animal that subsequent inoculation results in the development of a typical symptom complex and perhaps death, and in such cases the animal is said to be hypersensitive.4 This effect is apart from such phenomena as the neutralization of toxin by antitoxin and is most striking when the antigen is initially bland. While it may play some part in effective immunity, there is also reason to believe that hypersensitivity to the cell substance of bacteria may, in some instances, contribute to the pathology of the disease resulting from infection as, for instance, in acute rheumatism and arthritis, and hypersensitivity to nonliving antigens may also result in disease of noninfectious nature. Hypersensitivity is quite general and may take a variety of forms in both experimental animals and man. It may be divided here into three general categories:

(1) Anaphylaxis and related hypersensitivities

(2) Allergy and/or atopy

(3) Infection hypersensitivities

Of these, the first two are of the so-called immediate type in that reaction to the antigen occurs within a few minutes or hours, and the hypersensitivity is usually passively transferable by serum. They are distinguished from one another most obviously by the generally artificial nature of anaphylaxis, in a sense a laboratory phenomenon though it may occur under natural conditions, and by the spontaneous sensitization to environmental agents characterizing allergy and atopy. Infection hypersensitivities are somewhat set apart by their being delayed reactions, i.e., reaction to the antigen is delayed one to four days, and by their failure to be passively transferred by serum.

ANAPHYLAXIS18

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The sensitizing effect of an initial inoculation of antigen was observed by Richet in 1902 in a study of the immunization of

dogs with toxic extracts of the sea anemone. About this same time Theobald Smith noted the lethal effects of second and third inoculations of diphtheria toxin-antitoxin in the guinea pig when the inoculations were widely spaced, and in 1905 Otto found that the active agent in the mixture was horse serum. This was subsequently studied in considerable detail by Rosenau and Anderson, and the general picture is now quite clear.

Anaphylactic shock. If a guinea pig is inoculated with horse serum at intervals of perhaps four to eight days, it responds with the development of an immunity, and precipitating antibody appears in the serum. If, however, an interval of 10 to 12 days elapses between the first and second inoculations, the animal becomes hypersensitive as a consequence of the first inoculation. and the second is highly toxic. The state of hypersensitivity so induced by the sensitizing dose is designated anaphylaxis, and the reaction produced by the second, or shocking dose, is anaphylactic shock. This phenomenon of anaphylaxis is a laboratory artifact in that it results from the purposeful inoculation of antigen into the tissues, and shock is produced by the rapid inoculation of relatively large amounts of antigen, usually directly into the blood stream. It is artificial in that it occurs only under conditions set up by man, in experimental inoculation of animals and in serum therapy in

The sensitizing dose may be exceedingly small, as little as 0.000001 ml. of horse serum in the guinea pig, and varies with different antigens and different experimental animals. The shocking dose is considerably larger, 0.1 to 0.01 ml. of horse serum for the guinea pig. Quantitative studies with a pure antigen, crystalline ovalbumin, have indicated that somewhat larger doses may be required. Guinea pigs sensitized with 10 μ g. of ovalbumin could sometimes be fatally shocked with as little as $0.5 \mu g$., and doses of 1 µg, gave fatal shock in about 40 per cent of the animals. Within a certain range the severity of the shock produced is directly related to the size of the shocking dose; i.e., mild shock is produced by a small dose and lethal shock by a larger dose. The route of inoculation of the sensitizing dose is not

important. For some animals and some antigens several sensitizing doses may be required to develop hypersensitivity, but in any case the doses must be small and widely spaced. A single dose suffices to shock, and the route of inoculation is important. Intravenous and intracardial inoculation is most effective, and, in the guinea pig, which is the most readily sensitized and shocked of the usual experimental animals, intraperitoneal inoculation of the shocking dose is effective, but the reaction is delayed and larger doses are required.

Once established, the anaphylactic state persists more or less indefinitely but diminishes appreciably in the guinea pig after a few weeks. If the shock produced is not lethal, the animal is temporarily refractory, that is to say, is desensitized, but hypersensitivity reappears, in the case of the guinea pig after perhaps two weeks or less. Guinea pigs, rabbits, and dogs are the common experimental animals, but birds may be sensitized, while mice, rats, and monkeys can be sensitized only with difficulty and then irregularly. The symptoms and postmortem pathology are essentially identical within a given animal species regardless of the antigen used, but differ from one animal species to another.

The guinea pig. Following inoculation of the shocking dose the animal remains quiet for a few moments but very soon becomes restless, the fur becomes ruffled, and the animal rubs its nose and sneezes. Clonic and tonic convulsions set in, the animal falls to its side, respiration becomes labored, and the animal dies gasping for breath while the heart continues to beat. This symptom complex is produced in varying degree in nonfatal shock. The outstanding postmortem findings are the marked distention of the lungs and hemorrhages on the underside of the diaphragm. The lungs remain distended after the thoracic cavity is opened and when removed from the body and cut. The bronchioles are sharply constricted with the retention of air in the alveoli, and the immediate cause of death is suffocation. The contraction of the bronchioles is not prevented by vagotomy or curare and is muscular or immediately neuromuscular in origin.

The rabbit. The rabbit is less susceptible to anaphylactic shock than the guinea pig, and, in contrast, develops shock only

after a series of immunizing inoculations and after circulating antibody is demonstrable. Shock may be delayed and occur several hours after administration of the shocking dose. The symptoms and pathology differ sharply from those in the guinea pig, with absence of difficulty in respiration. The animal falls on its side, shows convulsive movements, and passage of feces and urine. Respiratory movements may continue briefly after the heart has ceased to beat. On autopsy the pulmonary dilatation found in the guinea pig is absent, and the most conspicuous and characteristic feature is the extreme dilatation of the right side of the heart. The immediate cause of death is heart failure resulting from constriction of the branches of the pulmonary artery and consequent dilatation.

The dog. Anaphylactic shock in the dog occurs in two stages. The first is characacterized by restlessness; the animal vomits and passes urine and feces and collapses with signs of extreme muscular weakness, and respiration is labored. The blood pressure falls rapidly as a consequence of capillary dilatation and stasis in the tissues. In nonfatal shock recovery from this stage is rapid, but in fatal shock the weakness is progressive, vomiting and diarrhea continue, convulsions may occur, and the animal becomes comatose and dies. On autopsy the outstanding feature is the congestion of the viscera and the pronounced distention and congestion of the liver, which is a consequence of constriction of the hepatic veins and injury to the liver cells resulting in edema from the transudation of fluid.

In addition to the characteristic phenomena noted above, anaphylactic shock also has common features which may be more or less pronounced in different animal species. Fall in body temperature is usual and leucopenia occurs which results from aggregation of leucocytes in the capillaries. Coagulation time of the blood is increased, usually most marked in the dog, as a result of the liberation of heparin rather than direct interference with the clotting mechanism, and serum complement is reduced. Congestion and hemorrhage of the gastrointestinal tract are common postmortem features.

Passive anaphylaxis. Anaphylactic hypersensitivity is passively transferable from sensitized female guinea pig to offspring and

by inoculation with serum from either sensitized or immunized animals. It can be produced with a high degree of consistency by inoculation of guinea pigs with immune rabbit serum and is a function of the amount of antibody, both precipitating and nonprecipitating, in the serum. As little as 0.03 mg. of rabbit or guinea pig antiovalbumin nitrogen suffices to passively sensitize guinea pigs, and univalent antibody is equally effective. The recipient is not immediately sensitized following the injection of antiserum and usually, though not invariably, a period of some hours elapses before sensitivity appears. If antigen is injected first, followed by the inoculation of antiserum, reversed passive anaphylaxis occurs, also usually some hours after inoculation of serum.

Function of haptenes in anaphylaxis. The specificity of the anaphylactic reaction may be, and probably frequently is, determined by haptenes. In general, sensitization can be accomplished only by complete antigen, but in a few instances simple compounds, including arsphenamine, picryl chloride, and 2.4-dinitrochlorobenzene, have been shown to sensitize guinea pigs, and sensitization to arsphenamine has been observed in man. Haptenes will usually not elicit shock, though some of the larger molecules such as azo dves will do so, but the inoculation of haptene specifically prevents shock by inoculation of the complete antigen. The role of haptenes in specificity is further shown by the use of conjugated antigens containing the same haptene but different proteins; thus, guinea pigs sensitized with a conjugated globulin can be shocked with the corresponding conjugated albumin.

Anaphylactic shock in isolated tissue.¹ It will be clear from the foregoing discussion that the occurrence common to anaphylactic shock in different animals is a contraction of the smooth muscle, predominantly in the bronchioles in the guinea pig, in the pulmonary artery in the rabbit, and in the hepatic veins in the dog, and in all three the gastrointestinal tract is affected. It is, then, of considerable interest that anaphylactic shock, manifested as a sharp and abrupt contraction, can occur in isolated smooth muscle. This was observed by Schultz in 1910, using portions of intestine, and studied in detail by Dale with uterine

horn, and is now generally known as the Schultz-Dale technique. The uterine strip is suspended in a bath of Ringer's solution with one end tied fast and the other attached to a kymograph, and the contractions are recorded when antigen is added to the bath. The reaction is exquisitely sensitive, a sensitized strip giving typical contraction in final dilutions of horse serum antigen as high as 1:1000 million, and sharply specific. All the essential features of anaphylaxis may be reproduced in vitro by this method, including active or passive sensitization of the uterine strip in vivo, passive sensitization in vitro, specific desensitization, etc. It is significant too that uterine horn from immunized as well as sensitized guinea pigs will respond to antigen with shock.

Mechanism of anaphylactic shock. It will be clear that anaphylaxis rests on an immunological basis and that shock is the result of antigen-antibody reaction. There is good reason to believe that the union is one of antigen with intracellular or sessile antibody. For instance, as just indicated, sensitization and shock may be produced in isolated tissue, and the incubation period in passive sensitization is regarded as necessary for the taking up of inoculated antibody by the cells. Desensitization consists of saturating, or nearly saturating, sessile antibody, and the protective effect of immunity is a prevention of antigen reaching intracellular antibody by union with serum antibody. Antigen-antibody complexes have pharmacological activity, and soluble complexes prepared with excess antigen will produce various manifestations of hypersensitivity, including anaphylactic reactions, skin reactions (see below), etc., in the normal intact animal or isolated tissue. 69, 98, 99

The manifestations of anaphylactic shock are closely similar to those produced by histamine and substances having histamine-like activity. In the guinea pig, for example, constriction of the bronchioles is produced which is indistinguishable from that of acute anaphylactic shock, and histamine and similar substances produce contraction of isolated smooth muscle in the Schultz-Dale reaction. It is generally believed that the antigen-antibody complex formed affects the cells and tissues in a way, as yet unknown, that results in the release of histamine-like substances. There is no reason to believe that antibody is fixed primarily or even pre-

dominantly in smooth muscle cells, but there is a high correlation between histamine sensitivity and the cells concerned in anaphylactic shock. Furthermore, the tissues affected in anaphylactic shock are those that show the highest content of histamine; there is some evidence which suggests that leucocytes are a source of histamine and, as indicated above, they aggregate in affected tissues in shock. In addition many, though not all, substances showing antihistamine activity appreciably or markedly modify anaphylactic shock; of these epinephrine and ephedrine are among the most effective.

While the activity of histamine and its resemblance to anaphylactic shock, and other manifestations of hypersensitivity, have been known for a long time, it is probable that other substances are concerned also. For example, antihistamines are only partially effective in inhibiting anaphylactic shock: the increased clotting time characteristic of anaphylaxis is not produced by histamine: refractoriness to histamine is not necessarily associated with refractoriness to anaphylactic shock, etc. Of other histamine-like substances, serotonin (5hydroxytryptamine) has also been associated with anaphylactic reactions. For example, both histamine and serotonin are released when antigen and antibody are added to a plasma suspension of normal rabbit platelets⁴⁶ and occur in rabbit blood during anaphylactic shock. The possibility that the predominant substance may differ from one animal species to another is suggested by the high content of histamine in the guinea pig lung, which contains little serotonin, and the reverse relationship in the mouse lung, 100 and the partial protection of guinea pigs but not mice by some antihistamines, while lysergic acid diethylamide, a serotonin inhibitor, protects mice.25 That more than one histamine-like substance may be effective in a single animal species is suggested not only by the incomplete protection given by antihistamines, but also by the observation that smooth muscle from an animal sensitized with multiple antigens reacts to each antigen successively; i.e., the contractile stimulant is not exhausted in one reaction.

A possible neurogenic mechanism in hypersensitivity reactions has been of interest in connection with the role of nerve conduction in the Schultz-Dale reaction. For example, stimulation by antigen or serotonin, but not by acetylcholine or histamine, is

inhibited by structural analogues of serotonin such as gramine, yohimbine, and bufotenine, suggesting that the antigen-antibody reaction may liberate serotonin. Treatment of the muscle preparation with botulinum toxin (Chap. Nine), however, blocks stimulation by antigen but not by serotonin, which has been taken to indicate that the toxin blocks the release of serotonin.

The nature of the pharmacological reactions mediated by the antigen-antibody union is clearly a highly complex one.

The Arthus phenomenon. The Arthus phenomenon is a local hypersensitivity reaction produced in actively and passively sensitized animals, which is closely related to, but not identical with, anaphylaxis, and which has been called a form of local anaphylaxis. If rabbits, for example, are injected repeatedly with antigen such as horse serum, a local reaction appears which becomes more and more intense as the inoculations are repeated. The site of inoculation first shows a transient swelling, but later the swelling and edema persist and progress to induration and local necrosis. This local phenomenon is a result of a general sensitization, since later inoculations need not be in the same site as earlier ones. The Arthus phenomenon appears to be confined to the rabbit among experimental animals, or at least is very difficult to produce in other animals, but occurs with some frequency in man, as during protracted series of inoculations with antiserum or an antigen such as rabies vaccine.

The rapidity of the reaction and its intensity are correlated with the amount of precipitable circulating antibody as well as with the amount of antigen inoculated. The reaction is an immediate one in that a cellular response is demonstrable histologically within an hour, but it does not become grossly apparent for several hours. The local antigenantibody reaction results in the deposition of the complex in the tissue adjacent to the site of inoculation, with preferential location in and on either side of the basement membranes of the small blood vessels.75 Such localization undoubtedly contributes to the vascular necrosis which is a prominent feature of the histopathology of the lesion. When a dye is inoculated with the antigen, it diffuses rapidly in the skin of a normal animal but is localized when the animal is sensitized; this reaction is referred to as passive cutaneous anaphylaxis (PCA) when used for the detection of skin-sensitizing antibody. 65 The Arthus reaction may also be reversed by the administration of antigen, followed several hours later by the local inoculation of antibody.

Serum sickness. The inoculation of man with antiserum, usually from the horse, produces in some persons a characteristic syndrome termed serum sickness. The symptoms include rash, often urticarial in nature, fever, joint pains, some edema, and swelling of the lymph glands regional to the site of inoculation, in combinations and emphasis that are variable from one individual to another. This reaction is to be distinguished from the febrile and local reactions commonly following the inoculation of foreign protein and is most often attributable to a hypersensitivity to horse serum irrespective of its antibody content, though essentially similar reactions may be produced following the inoculation of toxoids, vaccines, etc., as a consequence of hypersensitivity to constituent antigen. The incubation period may be as short as two hours or as long as 24 days, and most often is eight to 12 days. The reaction may follow initial inoculation of horse serum, but more frequently there is a history of prior inoculation. It is specifically antagonized by epinephrine, ephedrine, etc., and, like anaphylactic shock, is presumably a consequence of the sudden liberation of histamine in toxic amounts.

The hypersensitive individual gives an immediate reaction, *i.e.*, within 10 to 20 minutes, to the intradermal inoculation of 0.1 ml. of 1:10 dilution of horse serum or the instillation of a drop of horse serum in the conjunctival sac. In the first instance an irregular wheal surrounded by an erythematous zone constitutes a positive reaction, and in the second diffuse conjunctivitis appears in the hypersensitive individual.

Under certain circumstances, such as the prophylaxis of tetanus with antitoxin, desensitization may be carried out relatively rapidly. The usual procedures, after a preliminary test for sensitivity by intradermal inoculation of 0.02 ml. of a 1:10 or 1:100 dilution of the serum, include the administration of drugs such as diphenylhydramine hydrochloride, epinephrine, and corticotropin by intravenous drip, and, after 30 minutes or so, small amounts, 0.1 ml. of a 1:100 dilution, of antiserum are given subcutaneously. Successive inoculations can be given at relatively short intervals, 10 to 15

minutes, and doubled with each inoculation. Eventually 1 ml. doses can be given every 15 minutes until the total dose of antitoxin has been administered.⁸⁸

The question may be raised as to why serum sickness rather than anaphylactic shock occurs in the hypersensitive individual. In general there seems to be little tendency in man to generalized shock in this and other manifestations of hypersensitivity, and, as in dogs and monkeys, there is a tendency to localization of the manifestations of the reaction to the respiratory tract, the gastrointestinal tract, and the skin. A number of instances of typical anaphylactic shock have been reported in man though it is rare, and when generalized shock occurs it is often fatal.

ALLERGY AND ATOPY

Serum sickness is perhaps best regarded as a form of anaphylaxis in man, but only a small part of the manifestations of hypersensitivity are those of serum sickness; the remainder may be grouped under the general head of allergy. Like anaphylactic shock, an allergic reaction is a consequence of the union of antigen with sessile antibody. The antigen is often designated allergen and the corresponding antibody reagin because it was thought earlier that they differed from antigen and antibody. It is now generally recognized that there are no essential differences, but the terms persist even though a number of workers have urged that they be dropped.

There are a number of differences between anaphylaxis and allergy which are rather of degree than of kind, but which in the aggregate tend to distinguish them. Thus, allergy is naturally acquired while anaphylaxis is artificially produced; allergy is often a hypersensitivity to nonprotein antigens while anaphylaxis is only rarely so; allergic hypersensitivity is of long duration while that of anaphylaxis is limited; desensitization is usually difficult and incomplete in allergy and effective in anaphylaxis; and edema is a prominent feature of the allergic reaction and smooth muscle contraction a minor factor while the reverse is true in anaphylaxis.

Some workers divide the clinical allergies into two general groups: atopy (strange disease) or atopic allergy, and nonatopic al-

ALLERGY 409

lergy. The atopic allergies include the pollen and dander sensitivities resulting in hav fever and asthma and some of the food and drug allergies. Contact dermatitis and the remainder of the food and drug allergies make up the nonatopic group. The distinction between the two is quantitative rather than qualitative, and it is doubtful whether it is of any real validity, but it does have a certain clinical utility. It was first made on the basis of heredity, hereditary predisposition being an important factor in atopy, and it has even been postulated that in atopy initial contact with the antigen is not necessary for the development of the allergic state. On the other hand, in the nonatopic allergies there is almost always a history of contact with the antigen, such as continued exposure to nitrocellulose products in industry, and the hereditary predisposition seems to be of minor importance. There are other correlated characteristics. In atopic allergy the sensitivity is very high, desensitization is difficult and usually only partial at best, skin reactions are marked and specific, and considerable amounts of antibody are demonstrable in the serum. In nonatopic allergy the converse generally holds: i.e., sensitivity is low, desensitization is usually successful, skin reactions are weak and nonspecific, and little antibody is demonstrable in the serum. These distinctions are, however, purely relative.

Heredity. It has been a matter of some interest that inheritance of a predisposition to at least some forms of allergy is an important, perhaps often a determining, factor. The constitutional factor is a predisposition only, and contact with the antigen is essential to the development of the allergic state. Inheritance in man is frequently difficult to demonstrate, but detailed studies on familial association have made it highly probable that predisposition to allergic hypersensitivity is genetically determined. It has been suggested that the predisposition is determined by a pair of allelomorphic genes, H determining nonallergy and h allergy. The possible genotypes are HH or pure normal, hh determining allergy which develops before puberty, and Hh the normal transmitter in which allergy may develop after puberty. In confirmation of observations on man, the inheritance of predisposition to sensitization has also been shown in experimental animals. The significance of heredity in allergy has been said to distinguish this form of hypersensitivity from anaphylaxis, but it is not clear that this is a basis of distinction.

Forms of allergy. The allergic state is manifested in a variety of forms which are determined by two interrelated factors, the portal of entry of the antigen and the tissue predominantly affected, usually referred to as the *shock organ*. Thus the antigen may be inhaled, ingested, injected, or may simply make contact with the skin. The tissues affected are those of the upper respiratory tract, the gastrointestinal tract, and the skin. These combinations give rise to a number of commonly occurring, well-defined clinical types, *viz.*:

Hay fever. This is a seasonal allergy produced by the inhalation of pollens from trees, grasses, and weeds, and time of occurrence is determined by time of pollination, i.e., trees and grasses from late spring to midsummer and weeds in late summer and early fall. In the United States ragweed pollen is one of the most common offenders. Nonseasonal hay fever is a result of hypersensitivity to animal danders, orris root (a constituent of many cosmetics), and house dust. The mucous membranes of the upper respiratory tract are affected primarily.

Asthma. Essentially the same inhaled antigens are responsible for asthma as for hay fever, and in addition book bindings, straw, and similar materials are sometimes involved. The individual may become sensitive to bacteria of the normal flora of the upper respiratory tract, giving rise to so-called endogenous asthma. The shock organ is the lining and musculature of the bronchi in bronchial asthma, by far the most common type, and swelling of the mucosal lining and spasm of the muscles results in obstruction of the smaller bronchioles and consequent difficulty in breathing. Allergic asthma may also result from the ingestion of foods, such as eggs, milk, wheat, or various drugs. It may be noted that asthma is a symptom complex and not always allergic in etiology.

Dermatitis. Dermatitis of allergic etiology may result from contact with the antigenic substance or from ingestion or inhalation of antigen. The former is frequently referred to as contact dermatitis and is often an occupational disease resulting from repeated contact with substances such as lacquers, nitrocelluloses, glue, and the like. Ingested antigen not infrequently affects the skin as the shock organ, giving rise to a noninfectious eczema in infants and what is often called neurodermatitis in the adult.

Urticaria and angioneurotic edema. When the skin is the primary shock organ an inflammatory reaction accompanied by some degree of edema results. Urticaria, or hives, is the occurrence of whitish or pink elevations which come and go repeatedly within short periods, and is the most common lesion. In angioneurotic edema, or giant urticaria, edema is much more pronounced, and the lesions are large pale swellings which cover areas such as the eyelids, lips, and genitals. Hives and this edematous kind of lesion are frequently found together and most often result from hypersensitivity to ingested or injected antigen, i.e., foods and drugs.

The foregoing clinical types are characterized by the symptom complex produced, but types of allergy may also be differentiated on the basis of kind and portal of entry of antigen, viz.:

Drug idiosyncrasy. As indicated above, an allergic antigen may be a simple chemical compound, and when this is a drug the condition is a drug allergy or drug idiosyncrasy. The allergic sensitivity is to be differentiated from the sensitivity of a low tolerance for the drug; in the first instance the symptoms are those of the allergic reaction, while in the second they are produced by the pharmacological action of the drug. The drugs commonly involved are the barbiturate derivatives, salicylic acid compounds, phenolphthalein, opiates, sulfonamides, occasionally the antibiotics, the arsenicals, and others. These are either ingested or injected. The most common reactions are urticaria and dermatitis; bronchial asthma is less frequent.

Food allergy. The ingested antigens include food as well as drugs, and strawberries, milk, and eggs are the most common offenders. The shock organ in food allergy is usually the skin, and the most frequent lesion dermatitis or urticaria, and, less often, bronchial asthma. In addition, the gastrointestinal tract is often affected

directly with resulting disturbance.

Pollen and dander allergies. These antigens are inhaled, and the symptoms are almost always those of involvement of the upper respiratory tract, most commonly hay fever, and bronchial asthma somewhat less so.

Contact allergies. This is almost entirely the contact dermatitis noted above and is not only an occupational disease, but not infrequently results from cosmetics, the lacquers such as nail and hair lacquers, and powders containing orris root.

It will be clear from the foregoing that it is difficult if not impossible to generalize to a satisfactory degree the various interrelated forms of allergy, and the source of the difficulty is that they are not fundamentally different.

Allergic antigen and antibody. As indicated above, the allergic antigens or allergens (sometimes called atopens in the atopic allergies) are frequently nonprotein in nature. This is most obvious in the case of the drug idiosyncrasies in which synthetic substances, such as barbiturates, antipyrine, and the like, are clearly not contaminated with protein. Similarly, the substances responsible for contact dermatitis, such as lacquers, are protein-free. The more complex, naturally occurring allergens such as pollens, danders, and foods are, of course, not protein-free, but the active agent in some pollens is of lower molecular weight, perhaps 5000, than the usual antigenic proteins. There is no doubt that haptenic substances can produce the allergic reaction in a sensitized individual,

at least in experimental anaphylactic shock, but the question of sensitization is more difficult. High molecular weight nonproteins can act as complete antigens; for example, sensitization may be induced by pneumococcal polysaccharide. It is probable, however, that the relatively simple substances, such as the arsenicals, do not function as sensitizing antigens in themselves, and it is generally believed that they combine with body protein to give a conjugated complete antigen whose specificity is determined by the haptene, which functions as the sensitizing antigen. It is not clear, however, whether the shocking antigen is such a conjugated one or whether the haptene alone usually produces shock.

It may be noted here that allergic hypersensitivity to physical stimuli, heat, cold, and light, may occur. It is unlikely that such stimuli alter body proteins so that they become iso-antigens, and such allergies probably do not have an immunological basis.

On intradermal inoculation of soluble antigen, or its application as a patch on the intact skin, the sensitized individual gives a skin reaction characterized by local erythema and the appearance of a wheal. The skin reaction is more readily elicited and more specific in some allergies than in others. In general it is not satisfactory in contact dermatitis and some of the food and drug allergies. This kind of skin test is to be distinguished from tests like the Schick and Dick tests, in which the reaction is produced by diptheria or scarlatinal toxin and neutralized by circulating antitoxin, which have no relation to hypersensitivity.

Antibody⁸¹ is demonstrable in many of the allergies, particularly those grouped under the head of atopy. Its presence may be shown directly in some instances by complement fixation, but the usual measure is passive transfer of the sensitivity. If a small amount, 0.1 ml., of serum from a sensitized person is inoculated intradermally into a nonsensitive person and the antigen inoculated intradermally in the same area 24 hours later, a positive reaction occurs. The local sensitization may persist for four weeks or more. Such passive transfer cannot be made to guinea pigs but has been made to rhesus monkeys, and it is probable that a close phylogenetic relationship is essential. This passive transfer was described by Prausnitz and Küstner in 1921 and is known as

the Prausnitz-Küstner reaction. A reversed passive sensitization, analogous to reversed passive anaphylaxis, may be produced by inoculation of antigen first, and serum 24 hours later. In the usual terminology, the antibody which produces passive sensitization is the allergic reagin, and it appears to be exclusively IgA.

As in the case of anaphylactic shock, the allergic reaction is a consequence of the union of antigen with sessile antibody which results in the liberation of histamine or histamine-like substances. Allergic shock is antagonized by histamine antagonists, such as epinephrine and ephedrine, and a number of synthetic histamine antagonists, derivatives of dialkylaminoethoxy compounds (Benadryl and Decapryn), dialkylaminoethylamines (Antergan, Antistine, Pyribenzamine, Neohetramine, Phenergan), and dialkylaminoalkyl compounds (Trimeton, Thephorin).

HYPERSENSITIVITY IN INFECTION94

Bacterial cell substance may act as a sensitizing as well as an immunizing agent, and anaphylaxis may be induced with bacterial protein, though usually with much greater difficulty than with highly antigenic proteins such as egg albumin and serum proteins. Sensitization may also occur in experimental and naturally acquired infection but is highly variable, being especially prone to occur with some bacteria and not with others.

Hypersensitivity to the tubercle bacillus is the best known of the infection hypersensitivities and is typical of them. 64 It is demonstrable as a delayed (one to four days) local inflammatory skin reaction, the tuberculin reaction, to preparations of soluble antigen of the tubercle bacillus known as tuberculin. The tuberculin reaction is considered in detail elsewhere. This kind of reaction is somewhat different from the immediate wheal type of reaction, with respect to time of development and the nature of the dermal reaction, and in that it is not passively transferable. The generalized response also differs from the immediate shock of anaphylaxis: the inoculation of a tuberculous guinea pig with tuberculin in a dose sufficient to kill does not result in death until after some hours, and on autopsy the site of inoculation is congested, focal glands are swollen and congested, and focal reactions occur about tuberculous lesions which consist of areas of enormous dilatation of the capillaries. This is obviously different from fatal anaphylactic shock in the same animal. The nature of the antigenic stimulus inducing tuberculin-like sensitivity has been shown³¹ to be a protein combined with a wax fraction of the bacilli, the protein alone giving the usual immune response with the formation of precipitins.¹⁰¹

The infection hypersensitivities differ from anaphylaxis and allergy in that they are not passively transferable by serum. This kind of hypersensitivity is, however, passively transferable; the inoculation of a normal animal with cells from an induced sterile peritoneal exudate in a sensitized animal confers the hypersensitivity on the recipient. This transfer is analogous to the adoptive immunity produced by cell transfer described above.

Hypersensitivity is the outstanding immunological response to infection with a number of other microorganisms, and the skin reactions have been of some interest from a diagnostic point of view. In brucellosis, for example, a marked degree of hypersensitivity is developed and a skin reaction, a slightly raised edematous area, appears in about six hours after the intradermal inoculation of preparations of soluble antigen of Brucella. The preparations have been given various names such as abortin (from Br. abortus), melitin (from Br. melitensis), brucellin and brucellergen. Johnin, a preparation of Johne's bacillus, Mycobacterium paratuberculosis, is used in the diagnosis of Johne's disease of cattle.

Hypersensitivity occurs with regularity in glanders, and a skin reaction intermediate between the immediate and delayed types follows intradermal inoculation of a preparation of Actinobacillus mallei designated mallein. Similarly, in chancroid a hypersensitivity develops which is demonstrable as a skin reaction following intradermal inoculation of killed Ducrey's bacillus, and a hypersensitivity occurs in leprosy which results in a positive skin reaction to extracts of leprous tissue termed lepromin.

Hypersensitivity to fungi is also not uncommon; the mycids or secondary sterile lesions which occur in some kinds of dermatophytosis are manifestations of hypersensitivity. Infection with Coccidioides

immitis and Histoplasma capsulatum sensitizes, and a skin reaction to preparations designated coccidioidin and histoplasmin is demonstrable.

Hypersensitivity also occurs in lymphogranuloma venereum, and a skin test, the Frei test, with mouse brain antigen or yolk sac culture of the responsible organism, the latter marketed as Lygranum, has very considerable diagnostic value.

THE RELATIONSHIP OF HYPER-SENSITIVITY AND IMMUNITY^{3, 71, 95}

The status of hypersensitivity with respect to effective immunity to infection has been of very considerable interest in relation to tuberculosis. It was early supposed by Koch that the development of tuberculin sensitivity was indicative of immunity, and its significance in this respect is indicated by the well-known Koch phenomenon. If tubercle bacilli are injected subcutaneously into a normal and a sensitized (i.e., infected) guinea pig, the course of the subsequent infection is considerably different. In the normal animal the usual indurated nodule forms, which becomes necrotic to form a necrotic ulcer that persists while the infection spreads via the regional lymphatics, becomes generalized, and the animal dies. In the hypersensitive animal, however, an inflammatory reaction occurs, but there is no nodule formed; within a day or two the area becomes necrotic and finally sloughs without further spread, and the shallow ulcer heals rapidly. On the other hand, the inoculation of appropriate amounts of tuberculin markedly intensifies the cellular reaction about foci of infection, and the infection tends to spread rapidly. It would seem, therefore, that within limits the hypersensitivity is functional in effective immunity, but it may also be of very great disadvantage to the host. The whole question is of particular importance in relation to active immunization against tuberculosis.

As indicated earlier, hypersensitivity may take a variety of forms. Perhaps in large part because of clinical differences, there has been some tendency to regard the allergies as basically different, not only from anaphylaxis but from one another. It will be clear, however, that there are essentially

complete analogies running through all the forms of hypersensitivity, and the differences are more apparent than real. They arise from various factors such as the portal of entry of the antigen and its effectiveness in stimulating an immunological response and the shock organ affected. Thus, there is no real difference between the atopic and nonatopic allergies that is not accounted for on the basis of the relative efficacy of the sensitizing antigen – a high degree of sensitivity is associated with ease of demonstration of antibody and interferes seriously with desensitization. since it is difficult to give enough antigen to produce an adequate desensitization. Such considerations as these are the basis of the generally accepted belief that hypersensitivity is basically the same regardless of its clinical manifestations.

It is also clear that hypersensitivity is basically an immunological phenomenon, involving the stimulation of antibody formation and the union of antigen and antibody. The paradoxical situation arising from the contrast, say, between the neutralization of toxin by antitoxin, and the acquirement of toxicity by a bland antigen, is more apparent than real and a consequence of emphasis. Bronfenbrenner has illustrated the relationship by analogy with fire: the warmth, light, and other pleasing aspects of a grate fire are also present when the house burns down; both involve precisely the same mechanism, yet the emphasis on the consequences of the combustion is entirely different in the two cases.

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Chapter Sixteen

THE STAPHYLOCOCCI

The staphylococci are ubiquitous pathogenic microorganisms, are the commonest cause of localized suppurative infections, and are among the longest recognized of the pathogenic bacteria, having been characterized in the early 1880's largely through the work of Rosenbach. Nevertheless, they remain somewhat enigmatic and are less well known than the majority of bacteria. 30, 97

Morphology and staining. The coccus form tends to be much more uniform in size than the other morphological types of bacteria, and the staphylococci are consistently slightly less than 1 μ in diameter, and typically are more nearly perfect spheres than many cocci. Their most obvious morphological characteristic is their marked tendency to occur as masses of cells. This is a consequence of cell division occurring in three planes, coupled with a tendency of the daughter cells to remain in close proximity to give the characteristic appearance. These irregular clusters are three-dimensional; this is apparent on examination of wet mounts, but in the usual stained smear preparation. the clusters are flattened out to give the appearance of irregular sheets of cells. While some cells may be found singly, in pairs, or even very short chains, this characteristic morphology serves to identify the staphylococci except under special circumstances: e.g., it would be extremely difficult to differentiate staphylococci and many streptococci admixed in a smear.

These bacteria stain readily and deeply with the usual basic dyes in the simple staining procedure, and are strongly gram-positive, though rare gram-negative forms occur. They do not form spores, apparently do not have capsules except in the case of rare mucoid variants, and, although motile forms have been described, are almost invariably nonmotile.

Growth on agar mediums is abundant, and the colonies are opaque, smooth and glistening in appearance. Some staphylococci form lipochrome pigments which give the colonies a golden yellow or lemon vellow color, while others do not and are white. Although pigmentation is a variable characteristic, it is constant in primary isolates and has assumed considerable practical significance. The golden pigmented forms are Staphylococcus aureus (Staph. pyogenes var. aureus, Micrococcus pyogenes var. aureus), which are predominant almost to the exclusion of other forms in pathologic processes. The lemon yellow pigmented forms are Staph. citreus and appear to be largely saprophytic. The white forms are known as Staph. albus, with an attempt to distinguish between the varieties found on

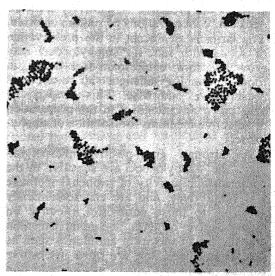


Figure 82. Staphylococcus aureus from pure culture. Note the characteristic clusters of the cocci. Fuchsin; × 1050.

the skin as a part of the normal flora as Staph. epidermidis, and Staph. pyogenes var. albus as the white staphylococci occasionally found in infectious processes; it is doubtful that such a distinction is valid or useful.

When cultured on blood agar plates, some of the staphylococci are β -hemolytic, and others are nonhemolytic; green or α -hemolysis does not appear to occur among the staphylococci. There is an approximate association between hemolytic activity and the formation of golden pigment, and the typical pathogenic form is hemolytic Staph. aureus, although nonpigmented or nonhemolytic, or both, forms are occasionally found in apparently causal relation to pathologic processes. Conversely, golden pigmented forms are not found exclusively in association with disease; some saprophytic forms are similarly pigmented.

Physiology. The staphylococci are relatively more resistant to heat, and to a certain extent disinfectants, than the vegetative forms of most pathogenic bacteria. While most bacteria are killed in 30 minutes at 60° C., higher temperatures and longer times, such as 80° C. for one hour, are often required for staphylococci. This assumes practical significance in the preparation of autogenous vaccines sometimes used in the therapy of persistent staphylococcal infections of the superficial tissues. They are also resistant to drying, may remain infectious for extended periods, and are able to grow in the presence of relatively high, 10 per cent, concentrations of sodium chloride. The latter is significant in the preservation of foods with salt, for staphylococci may grow and form enterotoxin (see below) in foods containing sufficient salt to act as a preservative otherwise. This property is also taken advantage of in the preparation of selective mediums for isolation of staphylococci.

As strongly gram-positive microorganisms, the staphylococci are sensitive to the bacteriostatic activity of triphenyl methane and other dyes, and are characteristically sensitive to those antibiotics effective on gram-positive bacteria, including penicillin and the broad-spectrum antibiotics such as the tetracyclines, but are quite insensitive to the antibiotics such as streptomycin whose antibacterial activity is confined to the gram-negative forms. They are especially prone to develop drug resistance (see

below), and some of these generalizations are frequently not applicable to current isolates.

Selective mediums. The majority of the coagulase-positive (see below) pathogenic staphylococci are able to grow in the presence of tellurite, reducing it to give graycolonies tellurite-containing black on mediums. In throat cultures on such mediums they may be confused with diphtheria bacilli, which are also resistant to tellurite and able to reduce it (Chap. Thirty). Tellurite mediums tend to be selective and have been useful in the isolation of staphylococci from heavily contaminated material such as fecal specimens. These staphylococci produce a lipase, or egg yolk factor (EYF),93 to give a zone of opacity in the medium around the colony when it contains egg yolk, and this characteristic has been combined with tellurite reduction in the development of selective isolation mediums.3,54 Mannitol (see below) and an acid-base indicator such as phenol red may be incorporated for differential, though not selective, purposes; and isolation mediums may be made selective by the inclusion of 10 per cent sodium chloride.16

Nutritive requirements. The staphylococci are not highly fastidious in their nutritive requirements, and grow readily on the usual meat extract-peptone mediums, but they grow more profusely on blood agar commonly used for isolation of the pathogenic forms. On semisynthetic mediums containing casein hydrolysate, nicotinic acid and thiamin are commonly required. and growth may be enhanced by the inclusion of additional bacterial vitamins, such as biotin, in these and simpler mediums. In chemically defined mediums, freshly isolated strains require a considerable number of amino acids, usually cystine, leucine, proline, valine, glycine, aspartic acid, phenylalanine and arginine, but the precise requirements differ from strain to strain. The bacteria can be "trained" by passage on successively simpler mediums to grow in the absence of most, or all, of the amino acids required by the parent strain.40 In general, freshly isolated strains of Staph. aureus are the most exacting in their nutritive requirements, and old laboratory cultures and Staph. albus less so.

Cultural characteristics. Lactic acid is the predominating end product of glucose fermentation, and small amounts of ethanol

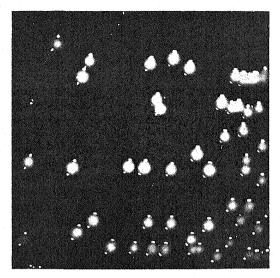


Figure 83. Colonies of *Staphylococcus aureus* on nutrient agar, 24-hour culture. × 3.

and carbon dioxide are formed. The fermentation of the conventional sugars and polyhydric alcohols is irregular and, for the most part, has no differential significance. Lactose, sucrose, maltose, glycerol, mannose, fructose, and erythritol are usually fermented; the fermentation of raffinose, inulin, and salicin is variable; and inositol. dulcitol, L-xylose, rhamnose, L-arabinose, adonitol, p-sorbitol, cellobiose, and dextrin are not fermented, and starch is not hydrolyzed. The fermentation of mannitol is considered to have differential significance in that it is fermented by the majority of coagulase-positive strains. Of the other conventional biochemical reactions indol is not produced from tryptophan, gelatin liquefaction is variable as is the reduction of nitrate. In sum, the biochemical reactions of the staphylococci, with the possible exception of the mannitol fermentation, do not provide a basis for their differentiation.

Antigenic structure. 66, 84, 85 The antigenic structure of the staphylococci of human origin has been studied in various ways, for the most part by agglutination, but also as the serological activity of soluble extracts in precipitin, gel diffusion, and passive hemagglutination reactions. Agglutination studies are complicated by the effects of blocking antigens, but these are minimized by using very young, four to six hour, cultures. Application of the conventional antigenic analysis technique has shown the presence of 30 antigens, arbitrarily designation.

nated by lower case letters, some heat-stable and some heat-labile. Typing is relatively difficult, and the slide agglutination test is considered to be much more reliable than tube titration.²¹

Soluble antigens, apparently largely carbohydrate in nature, prepared by extraction in hot dilute acid, allow the separation of the staphylococci into two groups, designated A and B; type A strains were originally thought to be pathogenic and type B nonpathogenic. It subsequently became clear that this differentiation is not clear-cut and these antigens do not occur in all strains. A protein antigen prepared by Verwey in 1940 has been reinvestigated and purified, and designated "protein A." This antigen, together with two polysaccharide antigens, polysaccharide A and polysaccharide 263, appear to be group-specific.

Attempts to correlate antigenic structure with other characteristics have not been successful. A correlation with phage type (see below) is believed by some⁸⁷ to occur, but not by others.⁸⁶ It has been suggested that serologic typing may be useful for strains untypable by phage.⁷⁰ There seems to be no correlation with virulence, and serological methods do not contribute to definition of pathogenic strains.⁴⁷

Phage typing. 12, 100 The host specificity of the bacterial viruses described elsewhere (Chap. Four) provides an additional means of characterization of bacteria, extending to strain differences. This method of differentiation was first applied to the typhoid bacillus (Chap. Twenty-one), allowing characterization within a single serotype, and has been extremely useful for epidemiological purposes. The application of bacteriophage typing, or phage typing, to the staphylococci has been largely the work of Blair and has been invaluable in providing a means of identification where the conventional biochemical and serological methods have thus far failed.

In brief, phage typing is based upon a collection of a group of phages of varied specificity but which in the aggregate will lyse the great majority of strains of bacteria encountered; these phages are numbered arbitrarily. While the maintenance of such standard typing phages requires technical precision, and test conditions must be defined, the typing procedure is simple. It is carried out by spotting the phages in appropriate dilution, and usually in two con-

centrations, on a petri dish culture of the bacterium inoculated to give a uniform film of growth. Sensitivity to phage is indicated by the clear zones of confluent lysis, grossly similar to plaques but not clonal in nature. When the bacterium is not infected by the test phage the film of growth is uniform.

Strain-specific phages are found but are rare, and phage typing is based upon a kind of spectrum or pattern of activity which is defined for the standard phages against standard test strains of bacteria. The number of such patterns is very large, impractically so, and it is recommended that for routine work a set of at least 21 basic phages should be used and grouped as follows:

Group I: 29, 52, 52A, 79, 80 Group II: 3A, 3B, 3C, 55, 71 Group III: 6, 7, 42E, 47, 53, 54, 75, 77 Group IV: 42D Miscellaneous: 81, 187

It is thus possible to characterize a strain of staphylococcus as belonging to one or another of these groups and phage types.

Such phage typing appears to be arbitrary in the present state of knowledge; *i.e.*, the nature of the susceptibility of a bacterium to infection with a bacterial virus is not yet subjected to precise definition. Among the enteric bacilli, the phage receptor on the bacterial cell may be related to the O antigen complex but, as noted above, a similar relation of contained antigen of staphylococci is quite uncertain.

Toxins. 10, 15, 75 It was apparent very early that cell-free filtrates of cultures of staphylococci are toxic on parenteral inoculation, and that extracellular toxin(s) is formed in considerable amount. The more obvious effects in experimental animals, commonly the rabbit, have been attributed to a necrotic, or dermonecrotic, toxin (dermonecrotoxin) and to a lethal toxin. In the case of the former, intradermal inoculation produces an intense inflammatory reaction, the center of the lesion becoming edematous and then necrotic about three days after inoculation. The prominent histologic feature is necrosis of the vessels, and the lesion so produced heals slowly. This activity appears to be closely similar among strains of staphylococci as indicated by protection by heterologous antiserums.

The lethal effect of the toxicity on the rabbit following intravenous inoculation may be a dramatic one. The incubation period is proportional to the dose, and may

range from as little as two minutes to as long as 24 hours with very small doses. The toxicity affects the heart directly and also the vascular supply to the lungs to give acute failure of the right heart, which is the immediate cause of death. On intracerebral or intrathecal inoculation, the toxicity acts upon the nerve cells, and death occurs very rapidly as a consequence of respiratory failure. These toxicities, and the activities of the α -hemolysin and Neisser-Wechsberg leucocidin (see below) disappear on treatment with formaldehyde to give a toxoid.

In addition to these, there are a number of other toxic activities. Among the more important are the staphylococcal hemolysins or staphylolysins; the enterotoxin; the leucocidins; and the enzymatic or kinase activities coagulase, hyaluronidase, and fibrinolysin or staphylokinase. Still other toxicities may be observed occasionally; for example, the formation of a scarlatinal or scarlatinal-like toxin has been observed, and gives a scarlet fever of staphylococcal etiology.³⁴

Staphylolysins. The pyogenic staphylococci are almost invariably hemolytic, and on primary isolation on blood agar the colonies are surrounded by a clear zone of β -hemolysis as described above. The hemolytic activity is also present in cell-free filtrates of cultures and consists of several distinct hemolysins, or staphylolysins.

The first to be differentiated were the α -and β -lysins. These differ immunologically, in the species of red cells lysed and in certain other characteristics. The α -lysin lyses sheep and rabbit red cells, but not human, guinea pig or horse cells. It requires magnesium or manganese ions and is inhibited by chelating agents.

The β -lysin acts on sheep but not rabbit cells, and has only slight hemolytic activity on human cells. It is produced by staphylococci of animal origin for the most part and is uncommon in human strains. It is also distinguished from the α -lysin in that it is a hot-cold lysin; i.e., except in very high concentrations, it does not lyse red cells when incubated at 37° C., but on chilling after such incubation the cells are lysed. It also requires magnesium ions, and the activity which disappears on treatment with chelating agents is restored by their addition.⁵⁹ In the warm phase incubation, the β -lysin potentiates the lysis of sheep cells by certain other hemolysins: that of group

TOXINS 419

B streptococci (Str. agalactiae) and the δ -staphylolysin.

A third hemolysin, the δ -lysin, differs immunologically from the α - and β -lysins, and, unlike them, lyses red cells from man, monkey, horse, rat, mouse, and guinea pig, as well as sheep and rabbit cells. 56, 57, 58 It differs also in that it reaches a peak in titer in 48-hour cultures rather than after 96 hours' incubation as in the case of the α - and β -lysins. Two other hemolysins have been described: the γ -lysin which closely resembles the α -lysin but differs immunologically and whose existence has been disputed; 30 and the ϵ -lysin which is reported to occur exclusively in nonpathogenic staphylococci.

The staphylolysins are not only hemolytic, but have other toxic activities. The α -lysin has been prepared in highly purified form, $^{8, 71}$ and the hemolytic, dermonecrotic, lethal, and leucocidal activities appear to be properties of a single substance. 67 The δ -lysin has also been found to be dermonecrotic and lethal in purified preparations. The β -lysin shows some toxicity on intravenous inoculation of rabbits, but is much less toxic than the α - and δ -lysins.

Of these staphylolysins, the α - and δ lysins predominate in strains producing disease in man and are highly correlated with the formation of coagulase (see below), more than 95 per cent of such coagulase-positive strains forming one or the other, and 82 per cent forming both. There is a similar high incidence of these lysins in coagulase-positive strains found on the skin; coagulasenegative strains from the same source do not form the α - and δ -lysins, but 95 per cent form ϵ -lysin. In contrast, α - and δ -lysins are less common in pathogenic strains of animal origin, occurring together in the absence of other lysins in only 10 per cent of strains, but the β -lysin is found in 88 per cent of such strains, and in combination with the α - and δ -lysins in 59 per cent. The incidence of these hemolysins is summarized by Elek and Levy.31

Leucocidins.⁴² The toxic effect of staphylococcal products on heterophils was observed prior to the turn of the century. Assay of this activity was put on a quantitative basis by the bioscopic method of Neisser and Wechsberg, using rabbit cells and measuring the toxic effect as the inability of the cells to reduce intracellular methylene blue, i.e., an inhibition of respira-

tion. Heterophils from man, mouse, and guinea pig are relatively resistant to this toxicity, and those from the frog almost completely resistant.

Human heterophils were used by Panton and Valentine, and the staphylococcal activity was found to inhibit phagocytosis. The Neisser-Wechsberg leucocidin is apparently identical with the α -lysin, and for all practical purposes affects only rabbit cells. In contrast, the Panton-Valentine (P-V) leucocidin, active on human as well as rabbit cells, is also antigenically distinct, and is formed by strains of staphylococcus which do not form α -lysin as well as those which do; i.e., it occurs independently. It reaches peak titer within 24 hours incubation, and is oxygen-labile and declines in titer as incubation is continued. It is separable by ion exchange chromatography¹⁰⁴ into two components, F and S, which act synergistically, and both components are antigenic⁴³ and may be toxoided with formaldehyde.41

These two leucocidins differ in their effects on heterophils. The α -lysin produces an agglutination of leucocytes and kills them as indicated by their staining with methylene blue, but the cells are not lysed. The P-V leucocidin produces a swelling of the cells to a spherical shape, with granules arranged around the periphery of the cell, and eventually the cell bursts and the granules are liberated.

A third leucocidin, designated leucolysin,⁴² has been described which differs from the P-V leucocidin in that it is thermostable, is active on the heterophils of all species tested except sheep, and is inhibited by cholesterol. It produces a nuclear swelling in the affected heterophils which are lysed in high concentrations of the activity. It is closely associated, if not identical, with the δ-lysin whose leucocidin activity had been described earlier.⁵⁵

Enterotoxin. The relation of staphylococci to the intoxication type of food poisoning described elsewhere (Chap. Twelve) was indicated by a number of observations, notably that of Barber in 1914 on acute gastrointestinal upsets associated with drinking milk from a cow with staphylococcal mastitis. It was not until 1930 that it was shown by Dack and his associates that such illness is produced by ingestion of cell-free culture of staphylococus strains which form enterotoxin.

This activity is relatively heat-stable and

unrelated to other toxicities of staphylococci. It appears to be formed only by coagulase-positive staphylococci, but not by all such strains. Enterotoxic strains are most commonly, 81 per cent of those typed, of phage types 6/47 or 42D,¹ but not all strains of these phage types are enterotoxic; the former is one of the commonest types carried by nasal carriers, and the latter a frequent cause of bovine mastitis. About 10 per cent of hospital strains were found to be enterotoxigenic in one study.⁴9 There appears to be no relation between the conventional biochemical properties of staphylococci and the production of enterotoxin.³²

Susceptibility to enterotoxin appears to be limited to man and certain monkeys. In man an acute gastrointestinal upset follows ingestion of the toxin in two to three hours and is characterized by projectile vomiting and diarrhea. Symptoms subside after a few hours, and recovery is complete.

A number of New World monkeys are susceptible and develop diarrhea and malaise, though usually not vomiting, following intragastric inoculation. The macacus monkey, Macaca mulatta, is the commonly used experimental animal. All monkeys do not respond, and susceptible animals may fail to respond after several, or even a single, such inoculation. 63 Chimpanzees have been reported to be much more susceptible.102 Purified toxin has an emetic effect on cats, presumably acting centrally,20 but the status of the kitten test for the detection of enterotoxin in culture filtrates is doubtful because peptone solutions also have a similar emetic effect.1

Enterotoxin is formed profusely in semisolid brain-heart infusion agar^{17, 38} and has been prepared as homogeneous protein with a molecular weight of 35,000 to 40,000. It is antigenic, and specific antiserum¹⁸ reacts in the gel diffusion test with as little as 1 μ g, per milliliter of purified toxin. The enterotoxin occurs as two immunological types, originally termed F and E, and now designated A and B respectively. 19 and a third, type C, has been described.7 The immunological methods are sufficiently sensitive that they may be used for the detection of enterotoxin, not only in culture filtrates, but also in appropriate extracts of foods.48 The safe

Coagulase. The ability of some bacteria, and staphylococci in particular, to

clot plasma has been described elsewhere (Chap. Nine). Staphylocoagulase has been of special interest because of its high correlation with virulence of staphylococcus strains of human and animal origin, and because of effects, such as protection of the bacteria against phagocytosis, antagonism of normal bactericidal activity of serum, etc., which plausibly relate to microbial virulence.

The activity is independent of the other staphylococcal toxins although associated with activities such as the α - and δ -lysins which also characterize virulent strains. It may be obtained cell-free although the results are somewhat erratic; the inconsistencies have been accounted for by the occurrence of bound and unbound coagulase.88 Cell-free coagulase^{33, 72} may be purified by salting out and ethanol fractionation.¹⁴ It is apparently protein in nature, containing little or no carbohydrate, and is readily inactivated by proteolytic enzymes. It is relatively heat resistant, to 60° C. for 30 minutes, and is only partially inactivated at 100° C. in the same time. It is antigenic, and serological studies28 have shown that at least four antigenic types, designated A, B, C, and D, are demonstrable; the several coagulases have been designated collectively as isocoagulases.⁷⁸ Toxoid may be prepared which is fully antigenic⁵⁰ but of, as yet, uncertain utility.

Coagulase activity in vitro is usually assayed in one or the other of two ways. One consists in mixing the bacterial culture with plasma, human or rabbit, and observing clotting. Calcium is not a factor and citrated or oxalated plasma may be used. This may be made more precise by using purified fibrinogen together with serum activator. For qualitative, and routine, purposes the plasma clumping test is used; the bacteria are suspended in a drop of plasma and coagulase-positive strains clump rapidly, giving the appearance of a slide agglutination test. The deposition of fibrin on the surface of the cells results in clumping, but the test does not precisely parallel the tube test. The presence of 1:10,000 Merthiolate, or inactivation of the plasma, interfere with the tube test but not the slide test. It has been suggested that the tube test measures free coagulase, and the plasma clumping test free or bound coagulase. Coagulase formation is also demonstrable as a halo of

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opacity around colonies of coagulase-positive staphylococci grown on agar medium containing fibrinogen.²⁶

The bactericidal activity of normal serum toward staphylococci is markedly inhibited by coagulase,²⁹ but the mechanism of the effect is uncertain; it is apparently not concerned with the respiratory metabolism of the bacteria.¹⁰⁵ Coagulase is not highly toxic on parenteral inoculation, but in sufficient doses produces a rapid fall in fibrinogen and extensive intravenous clotting, especially in the lungs, to produce rapid death.^{13, 94}

Hyaluronidase. 69,91 This enzymatic activity, which depolymerizes the ground substance of the tissues, hyaluronic acid (Chap. Nine), is formed by the majority of pathogenic staphylococci and is thus associated with other characteristics such as α - and δ -lysin formation, coagulase production, etc. Its enzymatic activity appears to be the same as that of hyaluronidases from other sources. While hyaluronidases of different origin are serologically different, even between groups of streptococci, staphylococcal hyaluronidase appears to be antigenically homogeneous.

Staphylokinase. 68 Fibrinolytic has been studied more intensively with streptococci than with staphylococci, but a considerable portion of staphylococci isolated from human carriers and from diseased tissue will disolve fibrin clots. The mechanism of staphylococcal fibrinolysis is similar to that of streptococcal fibrinolysis described earlier (Chap. Nine) in that the bacterial product is a kinase which activates a serum or plasma protease to give the lytic activity. It differs from streptokinase in that it acts on the plasma of animal species other than man, including the dog, guinea pig, and rabbit, but not the cow, and requires a longer incubation time. It is readily differentiable from staphylocoagulase in that it is heatlabile. A curious observation reported by a number of workers is that strains of staphylococci that produce β -lysin do not produce staphylokinase.

Variation. Like other bacteria the staphylococci undergo S-R dissociation. These variants have not been thoroughly studied with respect to the various toxins described above, and available information is limited. The characteristics used to differentiate the staphylococci, viz., pigmentation, toxigenicity in the broad sense, and biochemical

activity, are all subject to variation, and are considered in connection with classification (see below).

resistance. The staphylococcal Drug variation of by far the greatest practical importance is that in susceptibility to the antimicrobial activity of the antibiotics, and has been considered elsewhere (Chap. Seven) as an outstanding example of acquired resistance. The staphylococci appear to become drug-resistant more readily than most other bacteria. They may be made resistant to antibiotics by culture in the presence of successively increasing concentrations of these substances, and they become drug resistant under natural conditions. The appearance of drug resistance in strains isolated from pathologic processes has followed the introduction of the various antibiotics into general use, and the proportion of resistant strains found has continuously increased.

Penicillin was the first antibiotic to be used, and penicillin resistance appeared shortly after its introduction, followed by resistance to the tetracyclines, erythromycin, etc. Chloramphenicol resistance occurs also, but to a lesser extent, probably because this antibiotic has been used less frequently as a consequence of its association with damage to hemopoietic tissue and the occurrence of aplastic anemia following its continued administration. Drug resistance has taken the form of multiple resistance of individual strains of staphylococci, and strains resistant to more than one antibiotic are by now the rule rather than the exception. In fact, the incidence of drug-resistant staphylococci has reached a point at which the status of treatment and control of staphylococcal infections has, in the opinion of many, essentially reverted to that of the pre-drug era. This is of particular significance in view of the increasing prevalence and fatality of these infections.

Staphylococcal infections tend to be acquired in hospitals, and the proportion of drug-resistant strains found in carriers is much higher among hospital personnel than in the general population.^{35, 95} Precise figures vary from one area to another, and change with time in a single hospital, but in general 65 to 90 per cent of strains are penicillinresistant, perhaps 50 per cent tetracyclineresistant, 20 per cent erythromycin-resistant, etc., in hospitals, while the proportions are

much lower, possibly 5 to 15 per cent penicillin-resistant, in the general population.81

Extensive use has been made of phage typing in efforts to trace the sources of infection acquired in hospitals. The phage type 80/81 appears to be the most often penicillin-resistant, but this phage type is also a prevalent virulent one (see below). Retrospective studies, by subsequent determination of phage type of earlier isolates, of strains isolated over the years 1927 through 1947¹¹ have shown that the 80/81 type (described in 1955) was prevalent in this period, but that there was a relatively low incidence of phage types of Group III, which increased after the introduction of antibiotics. Subsequently strains of Group I increased, largely at the expense of Group III.99 A new type, 83A, has appeared61 and assumed epidemic spread in some areas;27,92 it tends to be untypable because of the interfering effect of lysogenic phages.2

Experimentally, and apparently also under natural conditions, resistant strains of staphylococci tend to revert to sensitivity, illustrated in the latter instance as a reduced frequency of resistant isolates. This tendency makes possible an effective approach to the problems posed by the occurrence of resistant strains, i.e., through control of the kinds of antibiotics used for therapeutic purposes. That such control may have practical consequences is illustrated in an experience with hospital populations in which therapeutic efficacy was improved by use of antibiotics indicated by the frequency of isolation of resistant strains.⁶

Penicillin resistance among the staphylococci is of two kinds. That induced by laboratory procedures is usually accompanied by morphological changes, notably the loss of gram-positiveness, and is associated with alterations in glutamic acid metabolism described earlier (Chap. Seven), and is of a temporary character in that reversion occurs with some facility. Penicillinfast staphylococci isolated from infection, however, usually show no morphological changes, and produce penicillinase. The significance of penicillinase production, i.e., resistance by virtue of decomposing the antimicrobial agent, is underscored by the sensitivity of penicillin-resistant strains of staphylococci to 2,6-dimethoxyphenyl penicillin (Staphcillin, Celbenin, Methicillin, BRL 1241), which is not degraded by penicillinase to the inactive compound penicillinoic acid.⁶⁵ Strains are beginning to be found,⁴⁵ however, which are resistant to such "synthetic" penicillins, but the mechanism of resistance is not yet clear. Penicillins are also decomposed by penicillin acylase (Chap. Seven) of bacterial origin, but this enzyme apparently plays no significant part in resistance *in vivo*, at least in part because of its high pH optimum.

Differentiation.25 Differentiation of species or types of staphylococci has centered largely around the definition of virulence of these bacteria in terms of associated characteristics. Virulence is assessed in two ways: the source of the strain of bacteria, i.e., whether or not from a disease process, usually suppurative, in which it appears to be the primary etiological agent; and pathogenicity for experimental animals (see below). Staph. aureus and Staph. albus appear to constitute the extremes in a continuous series of types, ranging from the virulent, pigmented, toxigenic, biochemically active forms, to the feebly pathogenic, almost saprophytic, nonpigmented biochemically inactive varieties.

The correlation between coagulase activity and virulence is very high, and the terms virulent and coagulase-positive have come to be almost interchangeable. Coagulase-positive staphylococci are almost invariably pigmented, e.g., Staph. aureus, usually ferment mannitol under both aerobic and anaerobic conditions, and dextrose, maltose, and glycerol; and liquefy gelatin. The mannitol fermentation is considered by some to have differential significance because it is highly correlated with coagulase; it has been reported that about 1 per cent of coagulase-positive strains are mannitol-negative, and about 8 per cent of mannitol-positive strains are coagulase-negative.64 Coagulase-positive strains practically invariably hemolytic on blood agar, and there appears to be a high correlation between the formation of α - and δ lysins and coagulase. In one study of 532 coagulase-positive strains isolated from carriers, all α -lysin-positive strains were also coagulase-positive, and only 4 per cent of coagulase-positive strains were α -lysinnegative, and all of 100 strains isolated from suppurative infections in man were α lysin-positive. Of 200 coagulase-positive strains isolated from man and assayed by the gel diffusion method, 82 per cent produced both α - and δ -lysin, 11 per cent produced α -, β -, and δ -lysin, 3 per cent α -lysin only, and 4 per cent δ -lysin only. In total, then, 96 per cent produced α -lysin, and 97 per cent δ -lysin. It will be recalled that the α -lysin is also a leucocidin, and the δ -lysin is both dermonecrotic and lethal. In contrast, of 77 coagulase-negative strains, none produced α -, β -, or δ -lysins, but 95 per cent produced ϵ -lysin, and the remainder were not hemolytic. On the basis of information such as the foregoing, then, it is possible to define tentatively a virulent staphylococcus as one that is coagulase-positive and produces α - and/or δ -lysins.

Classification.4,5 The formal classification of the staphylococci is, like that of many other bacteria, not completely satisfactory. Most workers accept three general groups of these organisms: (1) the Staph. aureus-Staph, albus series of strains or types with species status of the two extremes, virulent Staph. aureus and the relatively avirulent Staph. albus of the normal flora of the human skin, mouth, and upper respiratory tract; (2) a group of saprophytic staphylococci found in air, milk, etc., and including the lemon-yellow pigmented Staph. citreus, varieties forming pink to reddish brown pigments, and colorless forms closely resembling Staph. albus; and, (3) a group of obligate anaerobic staphylococci which are among the normal flora of the human body cavities, and one of which. Staph. aerogenes, is occasionally a cause of puerperal sepsis.

Pathogenicity.³⁰ Except in the case of staphylococcal pneumonias, these microorganisms enter the body either through the intact skin or following breaking of this barrier by trauma. The route of infection into the intact skin appears to be the hair follicles or sweat ducts. The staphylococcal infections commonly assume a localized form with a focus of purulent infection partially or completely walled off from surrounding tissues. This may be limited or may spread via the blood stream to give rise to secondary foci of infection in whatever tissues or organs the bacteria may lodge. Occasionally the infection may assume a fulminating bacteremic form.

The character of these infections is attributable, at least in part, to the properties of the toxic products of the microorganism. The close association of virulence with the formation of coagulase and the α - and δ -lysins allows a partial explanation of the

pathogenesis of the infections produced. It seems probable that the tendency of the focus of infection to be walled off can be attributed to the action of coagulase, and the purulent character of the lesion to the necrotic activity and killing of locally mobilized leucocytes by the α - and δ -lysins, 53 although an association of α -lysin with virulence under experimental conditions has been questioned.37 The deposition of fibrin on the surface of the bacteria appears to interfere with phagocytosis, and even phagocytosis, coagulase-positive after staphylococci tend to persist in viable form. 22, 76 The development of metastatic abscesses or focal infections, as in the bone giving rise to staphylococcal osteomyelitis, is a consequence of hematogenous spread of the infection in the form of thrombi and phagocytic cells containing viable microorganisms.

Pathogenicity for man. 74, 77, 90 Staphylococci are constantly present on the skin and in the upper respiratory tract. These are commonly the relatively avirulent Staph. albus, but virulent Staph. aureus occurs, especially in the upper respiratory tract.

CARRIERS. Nasal carriers are probably the most important single reservoir of staphylococcal infection of man.83, 101 increasing significance of infection with antibiotic-resistant staphylococci has resulted in a resurgence of interest in the carrier and the control of carrier-disseminated infection.⁷³ The spread of infection, airborne or by contact, is of particular concern in surgical wards and in maternity wards. The former is a prolific source of staphylococcal wound infection, and in the latter the newborn become infected within the first few days of life, and show a rate of perhaps 90 per cent on discharge. The infection rate in hospitals in this country has been found to be 9 to 13 per 10,000 patient days.³⁹ Examination of available data for the Scandinavian and Commonwealth countries and the United States over the period 1937-195980 has shown wide fluctuation in the carrier rates, from 20 to 60 per cent approximately, a reflection of the entry of penicillin into general use in the 1940's, and no general tendency to reduction; e.g., rates of 40 to 50 per cent are recorded for 1959.

SUPPURATIVE INFECTIONS. A transitory drop in resistance may be sufficient to allow local invasion and the establishing of a focus

of infection, perhaps acne or a simple boil, pemphigus or impetigo neonatorum, or a more or less extensive carbuncular condition may develop, possibly followed by the appearance of metastatic abscesses or bacteremia. Whether a focus of infection is initially established following penetration of the mechanical barrier, and the course of events following its establishment, are functions of the balance between the virulence of the microorganism and the natural immunity of the host. Thus a variety of lesions and diseases of the skin are of staphylococcal etiology, as are a large majority of cases of osteomyelitis and periostitis, many cases of otitis media and sinusitis and, much less often, urinary tract infections and the relatively rare staphylococcal meningitis. In general, suppurative inflammation, in whatever part of the body it may occur, is usually associated with the presence of staphylococci either in pure or mixed culture. When they occur in mixed infections, staphylococci may represent either primary or secondary invaders: it is often impossible to determine the sequence of events.

ENTERITIS.51 Staphylococcal enteritis came into increasing prominence with the general use of antibiotics, especially the broad-spectrum antibiotics given by mouth. Such therapy, or prophylaxis, favors the growth of resistant staphylococci in the bowel at the expense of the normal flora: staphylococci occur frequently in the bowel and are readily detectable in 10 to 15 per cent of persons with no enteric disease. The disease occurs more commonly in surgical patients given prophylactic antibiotic therapy. The severity of the enteritis is approximately related to the relative numbers of staphylococci present, and in severe cases the intestinal flora may be grossly abnormal, almost completely lacking in gramnegative bacteria and consisting largely of enormous numbers of staphylococci admixed with pus cells as observed in fecal smears. On autopsy, the enteritis is usually found to be a pseudomembranous type. It is not clear what part staphylococcal toxins, such as enterotoxin and the staphylolysins. play in the pathogenesis of the disease, or the role, if any, of staphylococci in ulcerative colitis. This kind of enteritis is to be distinguished from staphylococcal food poisoning (Chap. Twelve), although it is probable that the enterotoxins play a part in its pathogenesis.

PNEUMONIA.^{36, 46} Pneumonias of staphylococcal etiology, while not common, appear to be increasing and to be displacing a portion of pneumococcal and streptococcal pneumonias in pneumonia mortality,23 probably as a consequence of the continued susceptibility of the last two to antibiotics and the markedly increased prevalence of antibiotic resistant staphylococci. Staphylococcal pneumonias are probably almost always secondary, often to influenza;89 the alveolar edema of influenza is thought to favor staphylococcal infection. Two general types are recognized. One is a gradually developing cavitating pneumonia in which the initial lesion is a diffuse pulmonary mottling which coalesces to give consolidation and cavity formation. The other is a fulminating hemorrhagic pneumonia profound toxemia and is often accompanied by bacteremia. This second type is often secondary to influenza, and occurs in infants. 103 The bacteria may be found, frequently in almost pure culture, in sputum, empyema fluid, or lung puncture specimens, and in the blood when there is bacteremia. Intratracheal or intrabronchial inoculation of rabbits with cell-free filtrates has been found to result in consolidation, peribronchial areas of hemorrhagic congestion and necrosis, and blood vessel damage leading to ischemia of large areas of lung, a picture considered similar to that found in early cases of staphylococcal pneumonias in man.60

PREVALENCE. 60 Staphylococcal infections have increased in prevalence and fatality, practically displacing the pneumococcus as the immediate etiological agent in terminal infection, and these infections, together with tuberculosis and enteric infections, have become the predominant bacterial infections in this country. Epidemiological studies have shown that there is a marked association between particular phage types and virulence as inferred from the source of the strain.9 A large portion of virulent strains, to as much as 40 to 90 per cent in a given hospital, belong to the 80/81 complex. This complex includes strains which are also lysed by 52 and 52A phages, and the complex may be taken to include 80, 81, 80/81, 80/81/52/52A, and 80/ 52/52A.82 There is no evidence that the staphylococci have become more virulent; in fact, primary hematogenous osteomyelitis has declined in incidence. It is more probable that these infections have increased at the expense of others more susceptible to chemotherapy, and it is possible also that factors such as skin punctures for diagnostic purposes and the more general use of x-ray and cortisone may have contributed to a reduced resistance to infection with these microorganisms.

Pathogenicity for animals.^{24, 79} While man is considerably more susceptible to infection with staphylococci than the lower animals, naturally occurring enzootic infections in domestic animals are well known. and experimental animals may be infected. In horses and cattle Staph, aureus is associated with pathological processes and conditions similar to those it produces in man. Staphylococcal mastitis in cattle is not uncommon; the cattle are often infected by man, or the infection is carried from one cow to another by the milker, and infected cattle constitute a minor source of human infection. Staphylococcal infection of lambs is associated with tick bite and assumes an acute and a chronic form; the first is bacteremia with death occurring within 24 hours, and the second is characterized by the development of metastatic abscesses. Staphylococci have also been isolated from naturally occurring abscesses in birds.

Of the experimental animals, the rabbit is one of the most susceptible.44,62 Intravenous inoculation of 0.1 ml. of a 24-hour broth culture produces an infection that is fatal in four to eight days, and on autopsy abscesses are found in various organs, most commonly in the cortex of the kidney and in the walls of the heart. Usually infection does not occur in the bone marrow or periosteum, but it has been reported that osteomyelitis may be produced in young animals. If the bone is fractured or the periosteum is injured prior to inoculation, however, a sequence of events closely similar to osteomyelitis in man is produced. Ocular infections may be produced, but intraperitoneal infection is relatively unsuccessful. The mouse also is resistant to intraperitoneal inoculation, but following intravenous inoculation with approximately 40 million microorganisms, about 85 per cent of the animals die, and on autopsy abscesses in the kidney are the characteristic finding. Of the other common experimental animals, the guinea pig is relatively resistant to infection with staphylococci, and the rat and pigeon are highly resistant. In general, there seems to be no satisfactory animal test for the virulence of staphylococci.

Bacteriological diagnosis. The isolation of staphylococci is usually not a difficult matter. Blood agar is the medium of choice for isolation of the organism from purulent specimens, and on 24 hours' incubation Staph. aureus gives good growth of creamy, deeply pigmented colonies that are surrounded by the clear zones of β -hemolysis. The staphylococci are able to grow in the presence of 7.5 per cent sodium chloride, which is inhibitory to many bacteria, and selective mediums containing salt together with mannitol and sometimes phenol red as an acid-base indicator have been used for the isolation of staphylococci from specimens heavily contaminated with other bacteria.52,98

Examination of Gram-stained smears from typical colonies will show the characteristic morphology, and the coagulase test is routinely carried out, usually as the slide test in which the bacteria are suspended in plasma and observed under the microscope for clumping. Biochemical reactions, with the possible exception of mannitol fermentation and gelatinase formation, are uninformative. Differentiation of the lysins is carried out only for special purposes and most simply by inclusion of appropriate antiserums in a blood agar medium. The presence of enterotoxin in epidemiologically implicated foods, or the enterotoxigenicity of isolates, may be demonstrated immunologically by application of the gel diffusion test and specific antiserums as described above.

Immunity. Acquired immunity to infection with staphylococci is generally of a low order. Both natural and acquired immunity appear to be primarily cellular in nature, with phagocytosis of the microorganisms as the most significant factor. As pointed out above, the close association of coagulase with virulence suggests that this activity is an important element in the pathogenicity of the microorganism, but it is a poor antigen, stimulating only an irregular and low-grade immune response. The staphylolysins are good antigens, however, and the immune response to these and P-V leucocidin may account for the observations that antileucocidin is a significant part of effective acquired immunity. In addition, the nature of the infectious process is such as to minimize contact of the staphylococcal

antigens with both the antibody-forming cells and the circulating antibody. Microorganisms present in the abscesses produced by these bacteria are effectively separated from the blood stream; it is well known, for example, that staphylococcal osteomyelitis even of long standing does not result in an effective immune response, and, conversely, such lesions are not affected to an appreciable extent by circulating antibody or by drugs. Even on hematogenous spread of infection with the formation of secondary abscesses, many of the viable staphylococci are protected to a certain extent from antibody that may be present in the blood, for they are contained in leucocytes or within masses of fibrin.

Active immunization procedures, making use of vaccines, often autogenous, or cell-free filtrate containing staphylolysins tox-oided by treatment with formalin, are usually carried out on a therapeutic rather than prophylactic basis. Skin infections, such as pustular acne, are frequently difficult to treat, and active immunization has been of interest as a therapeutic measure. In general encouraging, though not striking, results have been obtained but may not appear for some months. The therapeutic use of antiserums has given disappointing results.

Chemotherapy. Sensitive strains staphylococci are susceptible to the antibacterial activity of the sulfonamides, penicillin, the tetracyclines, chloramphenicol, erythromycin, and other antibiotics active on gram-positive bacteria. The 80 per cent case fatality rate in staphylococcal septicemia was reduced to about 30 per cent with the introduction of penicillin, but subsequent development of drug-resistant strains has increased this over-all figure to 50 per cent or more. The problem of chemotherapy, of staphylococcal infections is, therefore, complicated by the prevalence of resistant strains, and strains of multiple resistance, and resistance to new antibiotics develops rapidly as they come into use. It is essential, therefore, that strains isolated from pathological processes be tested for sensitivity to a variety of potentially effective agents as a part of routine diagnostic procedure so that a basis for rational and effective chemotherapy may be provided.

When the strain is sensitive to an available chemoterapeutic agent, chemotherapy of staphylococcus bacteremia is strikingly successful, of localized infection in the form

of abscesses less so, and of osteomyelitis not at all without adequate surgery. In the last two, the microorganisms are partially or practically completely protected from the drug.

OTHER MICROCOCCI 96

A variety of micrococci, both pigmented and otherwise, have been described, most of which are saprophytic forms found in water and elsewhere in nature. A well-known representative of this group is *Sarcina lutea*, a coccal form producing a bright yellow pigment which derives its generic name from a tendency to form cubical packets of eight cells.

Micrococcus tetragenus (Gaffkya tetragena) is a parasitic coccus frequently found on the mucous membranes of the upper respiratory tract. It was discovered by Gaffky in 1881 in the pulmonary cavities in phthisis and has been found in pure culture in abscesses in animals and man, and often occurs in the healthy mouth. It is not uncommonly found in suppurations of the mouth and neck. It is also found in the empyema following pneumonia and in the pus of war wounds. This microorganism is probably of low-grade virulence and unable, as a rule, to invade the human tissues except

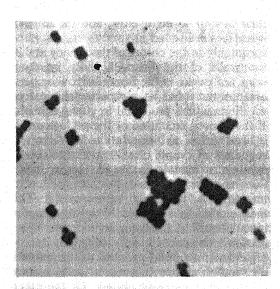


Figure 84. Micrococcus tetragenus; smear from pure culture stained with fuchsin. Note the relatively large size of the cells and the typical tetrad arrangement with the irregular clumps tending to be made up of tetrads. \times 1800.

when the resistance is lowered by some depressing influence, especially of the kind caused by the invasion of some other bacterium. White mice inoculated with *M. tetragenus* succumb to a rapidly progressing septicemia. Guinea pigs and rabbits usually show only a local affection. House mice and rats are relatively resistant.

Morphologically *M. tetrangenus* is distinguished by its occurrence in tetrads or groups consisting of four small oval cocci. It is gram-positive. In cultures the sheetlike arrangement is not always seen, but in the animal organism the flat tablets occur uniformly, and a rather heavy capsule surrounds the tetrad. On agar a confluent rough, elevated white growth is produced. Gelatin is not liquefied; milk is coagulated. Growth is slow and occurs at 20° and at 37° C., though better at the higher temperature.

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Chapter Seventeen

THE STREPTOCOCCI

The streptococci make up a relatively large group of pyogenic coccus forms characterized by an arrangement of the cells in chains. They are responsible for a variety of diseases of man and certain diseases of lower animals, and some are saprophytes found in milk and milk products. They were early observed in the pus formed in suppurative inflammatory conditions, and their frequent presence and pathological significance were first emphasized by Ogston, by Fehleisen, and by Rosenbach in the early 1880's. It is now well known that, in addition to the more virulent pathogenic forms, relatively harmless parasitic streptococci are more or less constantly present in the human throat and in the intestinal tract which assume a pathogenic role only under circumstances in which normal resistance is markedly reduced, and which may be regarded, for all practical purposes, as a portion of the normal flora of the human body.

Morphology and staining. Like staphylococci, individual streptococci are spherical and 0.8 to 1.0 μ in diameter. Some variation in size results from the character of the culture medium, and the individual cells are frequently appreciably smaller when the cultures are grown under anaerobic conditions. Smaller varieties, 0.4 to 0.8 μ in diameter, whose size is apparently a constant character, have been described. The typical streptococcus divides in only one plane, and the tendency of the cells to remain united results in the development of the characteristic chains that give these organisms their generic name. This tendency is apparently more pronounced between daughter cells following the first cell division, and the chains frequently have the appearance of chains of diplococci, with pairs closer to one another than to adjacent pairs. The firmness of the attachment is to some degree a strain characteristic, and some strains appear as relatively long chains while others show little more than two pairs of diplococci.

Earlier workers attached some importance to the length of chains formed and differentiated a supposedly more virulent Streptococcus longus and a less virulent Str. brevis. This distinction has little meaning, in spite of the fact that freshly isolated streptococci from pathological processes usually form chains of more than eight cells while those normally present in the mouth and throat usually develop only short chains. For instance, longer chains are generally formed during growth in liquid mediums; Str. lactis, a common inhabitant of milk, forms very long chains, and short-chain streptococci are not infrequently isolated from pathological conditions. The formation

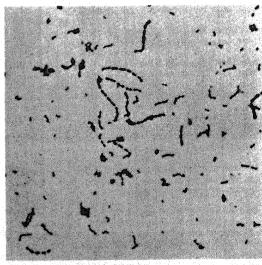


Figure 85. Streptococcus pyogenes. Recently isolated scarlet fever strain. Note the tendency to diplococcus arrangement in the chains. Fuchsin; × 1050.

PHYSIOLOGY 431

of chains of cells is in no sense absolute, however, and on microscopic examination of a smear of typical streptococci, single cells, pairs of cells, and occasional aggregates resembling staphylococci are found.

Streptococci are not motile under ordinary conditions of observation, but motile forms have been described from time to time.³⁴ The majority of strains are encapsulated, and in some the capsular material consists of hyaluronic acid, the substrate of hyaluronidase. So far as is known, these bacteria do not form spores, and the formation of pigment is relatively rare.

They stain readily with the usual bacterial stains. Almost all of the strains isolated from pathological processes in man are gram-positive, but gram-negative strepto-cocci are found, more commonly in suppurative conditions in lower animals than in man.

Streptococcus colonies on agar mediums are usually quite small, translucent, convex, entire, and slightly granular. The colonial form designated matt, with a ground-glass rather than a glistening or glossy surface, is the virulent form of β -hemolytic streptococci commonly encountered on primary isolation from infectious processes, but variant forms occur also (see below).

Physiology. As a group, the streptococci grow over a relatively wide temperature range, 10° to 42° C. Those of the pyogenic group, which is made up of human and animal parasites, have an optimum at 37° C. and are relatively restricted in range; those of the lactic group grow over 10° to 37° C.; and the viridans group grows from 37° to 42° C. and includes one thermophilic species which grows at 50° C. The majority of streptococci are facultative anaerobes, but there are a few obligate anaerobic varieties.

The streptococci are among the more fastidious of bacteria with respect to nutritive requirements. They will usually not grow on meat extract mediums, and growth is ordinarily poor even on infusion mediums but may be somewhat improved by the inclusion of phosphate buffer and a small amount, perhaps 0.1 per cent, of glucose. For the most part infusion mediums enriched by the addition of 10 per cent defibrinated blood, ascitic fluid, and similar substances are used, and a medium such as blood agar is satisfactory for routine culture of the pathogenic forms. Many strains are hemolytic on blood agar, some showing

the clear zones of β -hemolysis, and others a zone of greenish discoloration or α -hemolysis indistinguishable from that produced by the pneumococcus. Growth also occurs in milk which is curdled by some species because of the fermentation of lactose.

Streptococcus cultures may be preserved in serum broth or agar or infusion gelatin stabs in the refrigerator.

The relatively complex nutritive requirements of these bacteria have been defined to a considerable degree. Most strains require glutamine, riboflavin, pantothenic acid, pyridoxine, nicotinic acid, and biotin, together with 13 or 14 amino acids. The streptococci of group A also require nucleic acid derivatives. Some strains have been cultivated in mediums containing albumin or casein hydrolysate, supplemented with various amino acids and vitamins, and others in chemically defined mediums.⁵⁹

A wide variety of sugars are fermented and a number of polysaccharides are hydrolyzed. The chief fermentation product of glucose is lactic acid, and small amounts of formic and acetic acids and ethyl alcohol are formed. The hydrolysis of sodium hippurate and polymers such as inulin, starch, and dextrin has some differential significance, together with the fermentation of lactose, sorbitol, glycerol, mannitol, maltose, sucrose, and raffinose. Some strains liberate relatively large amounts of ammonia from peptone, and this characteristic also has some differential value. With rare exceptions, inulin is not fermented nor are the streptococci dissolved in ox bile or a 10 per cent solution of bile salt; these characteristics have considerable practical importance in that they serve to differentiate the α -hemolytic or green streptococci from the pneumococci.

The formation of toxic substances. The hemolytic streptococci produce a number of toxic substances. Certain of these appear to be intracellular and are found in sonic lysates of the bacteria, while others diffuse from the cells and are found in cell-free filtrates. In the former group, an intracellular hemolysin is formed which is demonstrable in sonic lysates⁸³ is distinct from the extracellular streptolysins (see below), and is lethal for the mouse.⁸⁶ Intracellular substances which are pyrogenic, produce rash, and give nodular lesions in the dermal connective tissue of rabbits have been studied at length.^{12, 14, 105} These activities appear to

be associated with the cell wall⁷⁷ and contain active polysaccharide,81 but are differentiated from the endotoxins of the gram-negative bacilli.11

The extracellular toxic activities include the streptolysins or hemolysins, streptokinase, hyaluronidase, the erythrogenic or scarlatinal toxin, leucocidin which kills heterophils that have ingested the bacteria, and possibly an enterotoxin in the case of certain of the α -hemolytic streptococci which are an occasional cause of foodpoisoning.63

Streptolysins. Two kinds of filterable hemolysin produced by streptococci were distinguished by Todd. They differ in that one, called streptolysin S, is sensitive to treatment with heat or acid, and the other which is designated streptolysin O, is inactivated by oxygen, i.e., is inactive in the oxidized state, and the activity may be restored by treatment with mild reducing agents such as sulfite. Both hemolysins are extremely labile at 37° C. and disappear rapidly after the first few hours of incubation.

Streptolysin O appears to be much the more important to the virulence of the hemolytic streptococci, and has a specific cardiotoxic activity which has been described by Bernheimer and his associates.5, 6, 43 Inoculation of the toxin results in the release of inhibitor, lipoprotein in nature, from the heart muscle of the frog resulting in sensitivity to the cardiotoxic action, and which is also released to occur in the serum albumin fraction and inhibits hemolysis in vitro.80 There is also evidence that streptolysin O has leucocidin activity. This toxin is antigenic, and antibody to it is regularly formed in streptococcal infections; the antibody titration has been put upon a standardized basis.25,98

Streptokinase.98 The hemolytic streptococci of Groups A, C, and G (see below) produce streptokinase, the plasmin activator that initiates the fibrinolytic dissolution of fibrin clots (Chap. Nine). It is most actively produced by strains of Group C and only irregularly and to low titer by strains of Group G. No special cultural conditions seem to be required for the production of the activity. There is some species specificity in that human, dog, and rabbit plasminogens are readily activated, but those of pig. horse, and cow are less readily activated, and guinea pig and sheep plasminogens are not activated by the usual concentrations of streptokinase. The activity is antigenic and antibody is produced, seemingly irregularly, in infected persons. The titration of the antibody is complicated by variation in the antiprotease and lytic factor concentrations in the test plasma, and, more important, streptokinase is not immunologically homogeneous and that produced by different strains of streptococci is often antigenically different. 107 Titration of such antibody levels has little significance in practice.

Hemolytic streptococci also produce streptodornase, a deoxyribonuclease which is apparently unrelated to virulence. It is not homogeneous, even within a single streptococcal strain, and has been separated electrophoretically into three components, A, B, and C; the last is inhibited by citrate and all three by chelating agents. 104 Streptokinase and streptodornase are available as commerical preparations (Varidase) for clinical application in the enzymatic debridement of necrotic tissue and dissolution of fibrous exudates.1

Hyaluronidase. The majority of hemolytic streptococci produce hyaluronidase, the enzymatic activity of which depolymerizes the gound substance of the tissues (Chap. Nine). Strains isolated from healthy throats produce as much hyaluronidase as those from mild or severe infections. The capsular substance of encapsulated strains of hemolytic streptococci of both Groups A and C consists of hyaluronic acid and such strains do not produce hyaluronidase although they are usually virulent. It is a plausible supposition that the production of this activity may be causally related to the ability of virulent streptococci to spread within the tissues, and in fact the production of hyaluronidase is increased in the presence of the substrate. The virulence of encapsulated strains and the lack of direct experimental evidence would seem to invalidate such an assumption. On the contrary, the production of hyaluronic acid, e.g., capsules, while apparently unimportant to experimental infection by the intraperitoneal route, appears to be essential to infection by inhalation. 15. 87

Erythrogenic toxin. The erythrogenic toxin is a substance which gives rise to a marked local erythema upon intradermal inoculation in man, and in larger amounts produces a generalized erythematous rash. A skin reaction may be produced in the rabbit, but, in general, laboratory animals are highly or completely resistant to it. The lethal dose for the rabbit is very large, 5 to 10 ml. of unconcentrated filtrate. This toxin is responsible for the rash of scarlet fever and is known as "scarlatinal toxin," or "Dick toxin" after its discoverers. It differs from the classic exotoxins in that it is relatively heat-resistant and some toxicity still remains after boiling for 30 minutes. It is antigenic and stimulates the production of specific antitoxin but not to the high titers readily obtained for diphtheria and tetanus antitoxin.

The formation of the foregoing substances by the streptococci is a characteristic of the group rather than of all strains of pathogenic streptococci. Thus all group A streptococci do not, produce erythrogenic toxin, and those that do not are incapable of causing scarlet fever. In general they are associated with virulence in that avirulent varieties are frequently nonhemolytic, nonfibrinolytic, and the like, while those found in pathological conditions and showing high virulence under experimental conditions are hemolytic, fibrinolytic, etc.

Hypersensitivity. Another factor associated with the ability of streptococci to produce disease is the development of a hypersensitivity to the cell substance of these bacteria during the following infection. Subsequent infections, then, result in allergic phenomena which may be of considerable importance in the disease produced. It seems probable that hypersensitivity plays a part in rheumatoid disease and arthritis of streptococcal etiology (see below).

Variation. Alterations in the morphology of individual streptococci are frequently observed in old cultures, with cells swollen to several times normal size. These and other changes in aging cultures have been interpreted by some workers as indicative of a complex life cycle, but it is more likely that such aberrant morphology is that of involution forms, *i.e.*, is degenerative in nature. The streptococci may, under appropriate conditions, occur as L forms and protoplasts.²⁴ These occur in Group C as well as Group A hemolytic streptococci.

Dissociative changes in colonial morphology are well known. A form designated as matt is the virulent form and distinct from the usual smooth and rough colony types. A mucoid colony type has been described which was still different. There are,

then, four recognized colonial types: smooth (glossy), rough, mucoid, and matt. The conversion from mucoid or matt to rough or smooth corresponds to the usual $S \rightarrow R$ dissociative change. The M antigen is present in the matt colony type, in fact derives its name from the association, but is deficient in the smooth glossy colonial type. Exceptions are not uncommon, and colonial morphology appears to be determined primarily by capsule formation; i.e., mucoid or matt colonies are formed by encapsulated streptococci and the smooth, glossy colony by strains which do not form them; the occurrence of M antigen is associated with capsule formation rather than contributing to colonial type. 114 Other immunological variation, not correlated with colonial form, may occur also, for there is some evidence that the agglutinative types are sometimes unstable and that on occasion the groupspecific polysaccharide may be lost (see below).

Variation in hemolysis is commonly reported in which β -hemolytic strains give rise to nonhemolytic or α -hemolytic variants. The alteration in hemolysis is to some degree an environmental effect in that anhemolytic variants may be hemolytic under anaerobic conditions, suggesting an inactivation of oxygen-labile hemolysin rather than failure to produce it; similarly α -hemolytic variants may be made β -hemolytic by including catalase in the medium or omitting reducing sugar, since the latter appears to inhibit hemolysin production by some strains of streptococci. Streptolysin O is generally regarded as responsible for blood plate hemolysis, but anhemolytic variants of β hemolytic strains have been observed which continue to form streptolysin O in liquid culture.

Drug resistance. Streptococcal infections are successfully treated with sulfonamides, penicillin, and the broad-spectrum antibiotics but may become resistant to these agents. Resistance may be induced experimentally in the usual way, and drug-resistant strains have been found to occur under natural conditions. As a group, the streptococci are much less prone to become resistant under natural conditions than are the staphylococci, and strains of acquired drug resistance do not occur often enough to present any practical problem. Although the β -hemolytic streptococci are relatively uniform in their susceptibility to the chemo-

therapeutic drugs, the α -hemolytic forms vary widely, but as a strain rather than an induced characteristic.

That resistant strains may occur on a relatively large scale was demonstrated during World War II, when sulfadiazine was used for the prophylaxis of streptococcal infection in certain military training camps.³¹ The resistant strain, or strains, that emerged spread in the military population and into the civilian population, but with discontinuance of the general use of sulfonamides when penicillin, and subsequently other antibiotics, became generally available, sulfadiazine resistance became of no importance and seems to have disappeared. On the other hand, the widespread use of penicillin, both generally and for the prophylaxis of rheumatic fever (see below), has not been accompanied by the general appearance of penicillin-resistant streptococci.

Classification. 87,88 The differentiation and identification of the streptococci is a matter of very considerable practical importance because of their etiological relation to a number of widespread diseases of man and domestic animals and, of equal importance, from the theoretical point of view. It has been and continues to be a particularly difficult matter, since neither the physiological nor immunological methods have been satisfactory. As a consequence there is basic disagreement among workers in this field as to what constitutes a species or variety and on what basis or bases differentiation should be made. Three general criteria have been used, viz., hemolysis on blood agar plate culture, biochemical properties, and immunological character as indicated by precipitin and agglutination reactions. Those concerned with the pathogenic streptococci make a tentative preliminary separation on the basis of hemolysis and define species and types on an immunological basis. Workers with more general interests tend to rely primarily on physiological characters, and this is the basis of the Bergey classification.

Hemolysis. The use of blood plate hemolysis was introduced by Schottmüller in 1903 and is especially convenient since blood agar is the medium of choice in primary isolation. On this basis three types of streptococci may be distinguished:

(1) The β -hemolytic streptococci, which produce a clear zone of hemolysis in the red opaque medium immediately surrounding the colony

(2) The α -hemolytic or green streptococci, which produce a zone of greenish discoloration in the medium about the colony which is considerably smaller than the clear zone of β -hemolysis

(3) The anhemolytic, indifferent, or γ streptococci, which produce no change in the medium

These distinctions have some validity in that the highly virulent streptococci isolated from pathological conditions are almost invariably the β -hemolytic variety. In the older literature these are grouped as a single species with the name Str. hemolyticus, and some workers further differentiated on the basis of the disease with which the strain was associated, viz., Str. scarlatinae (scarlet fever) Str. epidemicus (epidemic septic sore throat), Str. erysipelatis (erysipelas), etc. It is now quite clear that these distinctions are invalid in that identical streptococci may cause more than one clinical disease and that the same disease may be caused by immunologically distinct streptococci. At the same time, antigenic types of streptococci may be associated with one or another manifestation of streptococcal infection. such as the association of type 12 with acute glomerulonephritis; whether such associations as have been observed are more than fortuitous is not clear.

The β -hemolytic streptococci associated with diseases of lower animals do. however,

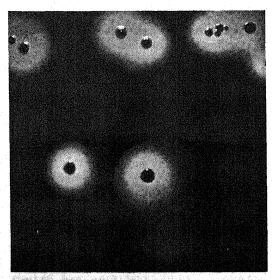


Figure 86. Streptococcus pyogenes. Pure culture on blood agar showing β -hemolysis. \times 5.

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show a high degree of specificity in the case of *Str. equi*, causing strangles in horses, and *Str. agalactiae*, causing mastitis in cattle, but not in that of the organism now known as *Str. zooepidemicus*, which causes a wide variety of suppurative diseases in animals, including mastitis in cattle.

The β -hemolytic streptococci of human disease are usually not found in lower animals, but may occasionally infect the udder of the cow, giving rise to milkborne streptococcal infection. Conversely, those of animal origin do not ordinarly infect man, though human infections with Str. zooepidemicus, while not common, are somewhat more so than is generally believed. An even greater heterogeneity in the group is indicated by the fact that some nonpathogenic forms are also β -hemolytic. It is clear that the inclusion of all these forms under the single species Str. hemolyticus is not justified.

A somewhat similar situation holds true with the α -hemolytic or green streptococci which have been grouped as a single species, Str. viridans. The green-producing group embraces such forms as the fecal streptococci or enterococcus group, those which normally inhabit the mouth and throat, and nonpathogenic forms. Some of the α hemolytic forms, especially those found in the throat and intestine, are able to set up disease processes if normal resistance is reduced, with the production of localized infections at the roots of teeth, in the heart valves in bacterial endocarditis, and other locations. These pathogenic forms differ rather sharply from the highly virulent β hemolytic streptococci, but again the entire group is too heterogeneous to justify inclusion in a single species.

The anhemolytic or indifferent streptococci are almost all saprophytic forms found in milk and various dairy products. The only pathological condition with which they have been unequivocally associated is subacute bacterial endocarditis,⁷⁸ in which they have been found in a small minority of cases. Here a variety of physiologically different types are included in a single group, and, as in the case of the other groups separated on the basis of hemolysis, it is too heterogeneous to allow lumping into the single species, *Str. anhemolyticus*.

Immunological differentiation. Largely

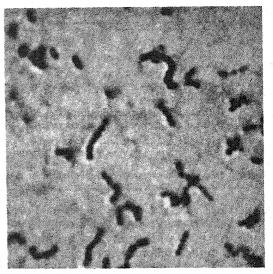


Figure 87. Group C streptococci stained with methylene blue to show capsules. Smear from serum broth. × 2400.

through the work of Lancefield and her coworkers, the pattern of antigenic structure of the β -hemolytic streptococci has been defined, but the viridans and anhemolytic forms are serologically diverse so that this approach has not been useful. By the extraction of soluble antigens and application of the precipitin test, Lancefield has demonstrated the presence of both group-specific and type-specific antigens in the hemolytic streptococci. The group-specific antigen, or C substance, is polysaccharide in nature and an integral part of the bacterial cell89, 90 rather than a capsular material, and is reported82 to be toxic in appropriate state of dispersion. On the basis of the specificity of this antigen five groups were originally described and designated Group A, Group B, and so on; additional groups have since been found, up to and including Group N. The biological singificance of these groups is indicated by the origin of the strains making them up, viz.,

Group A-Primarily pathogens of man

Group B-Found almost entirely in mastitis of cattle Group C-Primarily pathogens of lower animals

Group D-Found in cheese

Group E-Found in milk

The association of origin and immunological group is, however, not absolute. Streptococci of Group A are occasionally found in lower animals, producing mastitis in cattle

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Incidence of Streptococci of Groups A and C in Human Infections²⁰

		NUMBER	GROUP A		GROUP C		
	TYPE OF INFECTION	OF STRAINS	NUMBER	PER CENT	NUMBER	PER CENT	
	Scarlet fever	232	229	98.7	3	1.3	
	Tonsillitis and septic sore throat	52	52	100.0	0	0	
	Rheumatic fever and rheumatic arthritis	19	19	100.0	0	0	
	Puerperal sepsis	55	51	92.7	4	7.3	
	Erysipelas	51	48	94.1	3	5.9	
	Miscellaneous	143	136	95.1	7	4.9	
	Total	552	535	96.9	17	3.1	

for example, while those of Group C are found in man with some frequency as indicated in the accompanying table.

Type specificity among the streptococci was also indicated by the work of Griffith, who separated β -hemolytic streptococci isolated from human disease into 27 types by slide agglutination, and later added three more types. These were given arbitrary Arabic numbers, viz, type 1, type 2, etc. Following definition of the serological groups of Lancefield, it was found that these types are distributed over the groups; Group A included the majority, 23 in all, and types 7, 20, and 21 fell into Group C, and type 16 into Group D. 16

Lancefield has shown that the types within Group A are determined by two type-specific antigens. One of these, the "M" antigen, is a nucleoprotein located apparently very superficially in the cell wall.²⁷ It is destroyed by proteolytic enzymes, and may be digested by a soluble protease produced by streptococci, resulting in strains which cannot be typed by "M" antiserums. It has been purified to electrophoretic homogeneity but still contains non-type specific protein antigen.⁴⁸

The other type-specific antigen, designated the "T" antigen, is not as well known biochemically; it is resistant to proteolytic enzymes, but more recent work suggests that it is also protein in nature. The M and T antigens are independent and may occur in various combinations. Thus, types 10 and 12 contain the same M antigen but different T antigens, while types 15, 17, 19, 23, and 30 contain very closely related T antigens but distinct M antigens. In addition, strains are found which lack T antigen, and still others lack, or apparently lack, M antigen. An additional antigen, designated the "R"

antigen and similar to M antigen except that it is resistant to tryptic digestion and antiserums to it are not protective, was found first in type 28. Subsequently, it has been found, through use of the indirect bactericidal test,⁵⁵ in types 2, 3, and 48.⁴⁶, ⁴⁷

The nature and distribution of these antigens within the β -hemolytic streptococci of Group A, *i.e.*, Str. pyogenes, are shown in the accompanying tables.

While the separation of the streptococci of Group A is a complex matter, streptococcus typing has been rather generally applied, in part because it is of importance in the interrelationships, possibly phylogenetic, of the streptococci, and in part because it is of great value in epidemiological studies. 66 Many workers feel that slide agglutination is not completely satisfactory and favor the precipitin reaction; a method of carrying out the precipitin test in capillary tubes which is reliable and conserves serum has been developed.

Content of M antigen varies and is apparently related to the virulence of the strain, while T antigen usually remains constant within a given strain. Since type is determined largely by the M antigen, a strain which has small amounts of this antigen, or has temporarily lost it, may be difficult to type, though type may at times be inferred from the T antigen present. It may be progressively lost during infection coincident with increasing susceptibility to antibody activity,79 or more than one M antigen may occur as in the type 14 strains, found¹¹⁰ to contain a new M antigen, designated 51, and the mixed type as type 14/51; similar multiple type specificity occurs among the pneumococci (Chap. Eighteen). Rarely, the group-specific C polysaccharide may be apparently lost and replaced by a new poly-

Antigens of Streptococcus pyogenes*

		CHEMISTRY				
ANTIGEN SEROLOGICAL ACTIVITY		NATURE	PROPERTIES			
C	Group-specific	Polysaccharide	Polymer of N-acetylglucosamine and rhamnose; occurs in cell wall			
M	Type-specific	Protein	Alcohol-soluble; resistant to heating in dilute acid; destroyed by proteolytic enzymes; related to virulence, and antibody is protective			
Т	Occurs in several types but may be type-spe- cific	Protein	Resistant to proteolytic enzymes; unstable to heat in dilute acid, but resistant in slightly alkaline solution			
R	Occurs in types 2, 3, 28, and 48 and in strains of Groups	Protein	Destroyed by peptic but not tryptic digestion; unstable to heat in dilute acid, but stable to heat in dilute alkali			
	B, C, and G					

^{*}Modified from Lancefield: Streptococcal Infections. Columbia University Press, 1954, pp. 3-18.

saccharide, V polysaccharide, differing in that the ratios of rhamnose to acetylglucosamine are shifted from 1.6 for the C polysaccharide to 4.5. The change may be a superficial one chemically.⁵⁷

Streptococci falling into other Lancefield groups on the basis of the specificity of the "C" substance are also divisible into types. In Group B, the bovine mastitis streptococci, four main types and a number of subtypes have been differentiated. In this group there is but a single type-specific antigen, and it is polysaccharide in nature.

Distribution of Type-specific Antigens of Streptococcus pyogenes*

ТҮРЕ	M antigens	T ANTIGENS
1	M 1	T 1 (may be absent)
2	M 2	T 2 (may be absent)
3	M 3	T 3, T 1 (either or both may be absent)
15	M 15	
17	M 17	One common
23	M 23	Tantigen One common
19	M 19 j	One common Tantigen
30	M 30	T antigen
47	M 47	
12	M 12	T 10 and/or T 12 (may be absent)

^{*}Slightly modified from Lancefield: Streptococcal Infections. Columbia University Press, 1954, pp. 3-18.

Group C contains, in addition to the three Griffith types noted above, 10 additional types, five in strains of human origin, and five in strains from horses, making 13 in all. Here also there is but a single type-specific antigen, and it is protein in nature. Types have been differentiated in several of the other groups, such as Group D, 18 on the basis of a single type-specific antigen, polysaccharide in nature.

While the antigenic structure just discussed is largely that of the β -hemolytic streptococci, β -hemolysis is not invariably associated with the immunological character of these groups, and it has been found that certain of the enterococci, for example, contain these antigens. By and large, however, this antigenic structure does not extend far beyond the β -hemolytic group, and, as indicated above, immunological methods have not been useful in other than this group.

Physiological differentiation. There is some correlation between the immunological groups of the hemolytic streptococci and their physiological characteristics. Group A shows a reasonable degree of biochemical homogeneity. All produce β -hemolysis on blood agar and form soluble hemolysins, do not hydrolyze sodium hippurate, ferment trehalose but not sorbitol, do not reduce methylene blue, and seldom grow in 40 per cent bile agar. Group B is also more or less homogeneous differing from Group A in that sodium hippurate is

hydrolyzed and growth occurs in 40 per cent bile agar, but resembling that group with respect to methylene blue reduction and trehalose and sorbitol fermentations.

The streptococci of Group C are considerably more diverse and resemble Group A in that sodium hippurate is not hydrolyzed. Within the group there is some correlation between habitat and physiological character. Thus the strains isolated from strangles in horses are sorbitol-, trehaloseand lactose-negative, while strains that are pathogenic for man generally ferment sorbitol but not trehalose.

Physiological characteristics have been used exclusively in the Bergey (1957) classification of the streptococci and the species differentiated on that basis are indicated in the accompanying abridged key.

Most of these species are generally accepted, and it will be clear from the foregoing discussion that all members of Group

I. Facultative anaerobes

A. Pyogenic group

- 1. Sodium hippurate not hydrolyzed
 - a. Lactose fermented
 - Lancefield Group A i. Sorbitol -, trehalose +

Streptococcus pyogenes

ii. Sorbitol +, trehalose -Lancefield Group C

Streptococcus zooepidemicus (animal pyogenes)

- b. Lactose fermentation variable
 - i. Trehalose -

Streptococcus equi

ii. Trehalose +

Streptococcus equisimilis

2. Sodium hippurate hydrolyzed

Lancefield Group B

Streptococcus agalactiae (mastitidis)

- B. Viridans group
 - 1. Lactose fermented
 - a. Does not grow at 50° C.
 - i. Starch not hydrolyzed, not tolerant of bile

Streptococcus salivarius

Streptococcus mitis

ii. Starch is hydrolyzed, bile-tolerant

Streptococcus bovis

b. Grows at 50° C.

Streptococcus thermophilus

2. Lactose not fermented

Streptococcus equinus

- C. Lactic group
 - 1. Maltose +, dextrin +, ammonia from peptone

Streptococcus lactis

2. Maltose -, dextrin - (usually), no ammonia from peptone

Streptococcus cremoris

D. Enterococcus group

Lancefield Group D

- 1. Not β-hemolytic
 - a. Does not hydrolyze gelatin

Streptococcus faecalis

b. Gelatin hydrolyzed

Streptococcus liquefaciens

- 2. B-hemolytic
 - a. Mannitol +, sorbitol +

Streptococcus zymogenes

b. Mannitol -, sorbitol -

Streptococcus durans

II. Micro-aerophilic or obligate anaerobes

Streptococcus anaerobius

Streptococcus foetidus

Streptococcus putridus

Streptococcus lanceolatus

Streptococcus micros

Streptococcus parvulus

Streptococcus intermedius

Streptococcus evolutus

A are known as Str. pyogenes, the immunological varieties within the group being types of Str. pyogenes. Two species are included in Group C. The name Str. zooepidemicus is a relatively new one introduced in the Bergey (1948) classification; it does not replace another name, but rather gives species status to the animal pathogens of this group formerly casually known as "animal pyogenes" which rarely occur in man. The other species, Str. equisimilis, includes those streptococci formerly known as "human C" and which are uncommon in lower animals. Of the β -hemolytic streptococci found in man, 95 per cent or more are Group A and therefore Str. pyogenes, and the remainder, Group C, are Str. equisimilis, differentiation being made by the precipitin test, using group-specific antiserums. Of the green streptococci found in man, the most common are Str. faecalis of the enterococcus group and Str. mitis and Str. salivarius of the viridans group. So far as the etiology of streptococcal infections in man is concerned this differentiation 109 and that of Str. faecalis and Str. faecium are unimportant. The identification of streptococci which do not fall into the Lancefield groups is a matter of detailed biochemical study.

Pathogenicity for animals. As indicated above, certain of the streptococci are responsible for specific diseases of domestic animals. Str. equi is the cause of strangles in horses, a suppurative infection of the upper respiratory tract that is characterized by abscess formation in the throat and submaxillary region. This species is apparently not pathogenic for man. Other strains of Group C infect horses, causing respiratory catarrh and suppurative lesions in various parts of the body.

The most common cause of streptococcal mastitis in the cow is *Str. agalactiae*, which produces a chronic infection that is, as a rule, more common in older cattle. The infection is probably spread by the hands of the milker for the most part. Mastitis may also be caused by *Str. pyogenes* and may result in the spread of milkborne streptococcal infection of man; it is probable that the source of infection is man and that *Str. pyogenes* does not occur naturally in the cow.

Various other suppurative conditions, often involving infection of lymphatic tissue in animals such as the dog and sheep, are of streptococcal etiology. Some are Group A streptococcal infections, as in the

case of epizootics which have been observed to occur in wild rodents⁴ and laboratory mice,³² but usually are β -hemolytic streptococci of groups other than Group A; there are a few reports of spontaneous infections of lower animals with green streptococci.

Str. pyogenes is pathogenic for most laboratory animals, including the mouse, guinea pig, and rabbit, but different strains vary widely in virulence for different animals; virulence may, of course, be enhanced by animal passage. Intravenous inoculation of virulent strains results in fatal septicemia; suppurative peritontis developing into septicemia follows intraperitoneal inoculation, and subcutaneous inoculation produces an abscess from which the infection may or may not spread. Pneumonia may be produced in rats by the intrabronchial inoculation of the bacteria suspended in mucin. Str. agalactiae is of low virulence for experimental animals, and Str. equi is highly virulent only for the mouse. The green streptococci are also relatively avirulent for laboratory animals but may produce local infections on intravenous inoculation following injury; such techniques have been used to produce experimental arthritis lesions in the rabbit.

Pathogenicity for man. 56 The streptococci are responsible for a wide variety of diseases of man, perhaps a greater variety than any other kind of bacteria, and, in addition to being the primary cause of disease, they have a marked tendency to occur in mixed and secondary infections with other pathogenic bacteria. In general, the streptococcal infections are characterized by suppurative lesions and very often manifestations of toxemia, the latter taking the form of the so-called nonsuppurative complications of the infection and including fever, arthritis, carditis, and nephritis. They vary in the extent to which the body is involved, from local infections such as abscesses of the various tissues, including mucous membranes, joints, and serous membranes, infection of the muscle or cellulitis which simulate gaseous gangrene, suppurative processes in all kinds of wounds, to those generalizing to pyemia or septicemia. Some streptococcal infections have no distinctive clinical manifestations; thus an abscess caused by streptococci is not distinguishable from one of staphylococcal etiology. Others, however, such as erysipelas, streptococcal sore throat, scarlet fever, and the like, have to a greater or lesser degree some distinctive clinical character.

There are appreciable differences in the character of streptococcal infection in the child and in the adult, and the lower age groups are relatively the more susceptible. In the infant the infection tends to be a prolonged, low-grade infection with frequent suppurative complications, but rheumatic fever and nephritis seldom follow. In older children and adults the infection tends to be acute and self-limited and with nonsuppurative complications.

The β -hemolytic streptococci are by far the most virulent and, as indicated above, the great majority of those infecting man are of Group A, Str. pyogenes. The small proportion of infections with Group C streptococci are not distinguishable from those of Group A except by isolation and immunological typing of the etiological agent. Human infection with Group B streptococci is described from time to time^{45, 53} but is

The pathogenicity of these bacteria is accounted for to a considerable degree by the soluble toxic substances they produce.² The most clear-cut example is that of the relation of the erythrogenic toxin to scarlet fever, but it is probable that other toxic substances also play a part in the development of the pathological condition.

The association of fibrinolysin with virulence seems definite, so much so that this activity has been termed invasin by some workers.

The role of the streptolysins in the development of the pathology of streptococcal infections is not too clear. Both streptolysin S and streptolysin O are toxic for experimental animals, the former causing death through intravascular hemolysis. The mechanism of the lethal action of streptolysin O is not known; it may possibly be related to the cardiotoxic action of this substance described by Bernheimer. There is some reason to believe that the destructive effect of streptococcal filtrates on polymorphonuclear leucocytes, attributed to the presence of a leucocidin, is closely related to or identical with streptolysin O, but in any case this activity may make up a significant part of the invasive and pathogenic properties of the streptococci. The presence of the type-specific M antigen is associated with virulence, and antibody to it is protective while antibody to the type-specific T antigen is not. Hyaluronic acid capsules seem to be at best a minor component of virulence among Group A streptococci but appear considerably more important in this respect in Group C.

In general, then, while knowledge of the mechanisms of the pathogenic action of the β -hemolytic streptococci is far from complete, there is a considerable body of evidence which indicates that the production of disease is due at least in part to the action of the several toxic substances formed by these bacteria. As indicated earlier, strains of streptococci differ with respect to the kinds and amounts of such substances that they produce, and this variability accounts in part for the differences in disease they may produce. Thus, both strains which produce erythrogenic toxin and those which do not may produce septic sore throat, but only the former can produce scarlet fever also. These are not the only factors, of course, and the route of infection and immunity of the host are significant also; for example, wound infection and streptococcal sore throat involve different routes of infection, and an erythrogenic toxin-producing strain can produce sore throat but not scarlet fever in the immune.

Streptococci other than the β -hemolytic varieties are much less virulent.²¹ The α hemolytic forms constitute a portion of the normal bacterial flora of the mouth, upper respiratory tract, and intestinal tract. It is probable that they seldom initiate infection of the healthy tissues, but when natural resistance has been reduced they may be able to set up low-grade, essentially localized, infections such as focal abscesses in the teeth and gums.78 They are the most common cause of subacute bacterial endocarditis but, while the condition is a serious one, the infection shows little or no tendency to spread throughout the body in spite of the frequent presence of the streptococci in the blood stream.

Pneumonias of α -streptococcal etiology occur from time to time. ¹⁹ The disease tends to assume a chronic relapsing condition, with pleuritis and lung infiltration, and a tendency to bilateral involvement. It resembles viral pneumonias superficially, but differs in that there is a marked leucocytosis. A few instances of α -hemolytic streptococcal meningitis have been reported, ³⁸ but are rare.

Streptococcus MG. The nonhemolytic, or anhemolytic, streptococci are largely saprophytic forms, often found in milk and dairy products, and have been associated with disease only in rare cases of subacute bacterial endocarditis. They are occasionally isolated in atypical pneumonias, which are most often of varied viral etiology, but apparently are not causally related to the disease. One such strain, designated streptococcus MG, has been isolated from the sputum in a series of cases of primary atypical pneumonia, and proved to be biochemically and antigenically homogeneous.61 About half the cases of such disease develop specific agglutinins for this strain of streptococcus, and these are known as MG agglutinins. This antibody response is distinct from the cold hemagglutinin that also occurs in primary atypical pneumonia, and the two kinds of antibody have empirical diagnostic utility although so far as is known they have no bearing on the Mycoplasma etiology of this disease (Chap. Twenty-seven).

Epidemiology of streptococcal disease. The primary source of pathogenic streptococci is the human being who carries these bacteria in the upper respiratory tract. The infection may not be associated with symptoms, and carrier rates of 4 to 25 per cent to as much as 40 to 60 per cent in schoolchildren, with cumulative percentages as high as 75 to 90 per cent,68 have been reported. It is estimated that overt streptococcal infections occur in all individuals from time to time, at intervals of perhaps two to five years, but the incidence of subclinical infections is not known other than in restricted groups. Those with overt symptoms of disease such as tonsillitis, pharyngitis, sinusitis, and scarlet fever are prolific sources of infection. While streptococci may be present in the saliva as well as in the throat, and discharged by sneezing, coughing, and contamination of the hands, the nasal carrier is by far the most dangerous and contributes very large numbers of streptococci to his environment. Not only is the carrier the source of infection, but the streptococcal disease may take a variety of clinical forms. In an epidemic of scarlet fever, for example, the cases of pharyngitis and rhinitis are as important as those of frank scarlet fever, i.e., those showing a rash, in the spread of the infection; in fact it is customary to record the incidence of scarlatinal rash in a given epidemic rather

than differentiate scarlet fever from other streptococcal infections of the upper respiratory tract.

The transmission of streptococci from the infected person to the susceptible individual is in part a matter of direct contact and in part one of contamination of the environment.51,65 Direct contact include inhalation of infective droplets expelled from the nose and mouth, hand-tohand contact, etc., while contamination of the air with droplets too small to settle, and through air and droplet infection the contamination of dust. Direct contact with the hands no doubt accounts for wound infection, puerperal fever, infection of the udder with Str. pyogenes to produce mastitis and milkborne streptococcal disease, and possibly infection of the upper respiratory tract to a certain extent. Most upper respiratory tract infection is airborne, 29, 30 either directly or through the agency of resuspended infected dust. The importance of the last is very great, and dust suppression measures such as oiling of blankets in hospital wards sharply reduce the incidence of streptococcal infection.

Although some streptococcal diseases are reportable, the incidence of streptococcal infection can only be estimated; probably about seven million infections occur each year in this country.

Immunity to streptococcal infection. Antibodies to the streptococcus cell substance and to the antigenic soluble products of these organisms are formed following immunization or infection. In the case of Str. pyogenes, the former include the type-specific M and T antigens as well as the groupspecific C substance. In the latter group are the erythrogenic toxin, streptolysins S and O, hyaluronidase, and fibrinolysin. Antibody formation has no diagnostic utility in acute streptococcal infections, in part because there is not sufficient time for antibody formation during the course of the disease, at least in its early stages, and in part because isolation and, if desirable, typing of the infecting microorganism are relatively simple. Antibody titration is useful for diagnosis in retrospect, i.e., in associating streptococci with diseases such as rheumatic fever and arthritis, and for the determination of susceptibility to scarlet fever on the basis of antibody to the erythrogenic toxin.

The immunized animal responds to the

cell antigens of streptococci with the production of precipitating and agglutinating antibody, and the use of such antiserums makes possible immunological grouping and typing. The immune response of man to these antigens during the course of infection appears more often to take the form of the development of a hypersensitivity, especially in the rheumatic diseases, and the immunological response may be measured by intradermal inoculation of soluble streptococcal antigens. More often serum is titrated for antibody to streptolysin or fibrinolysin by in vitro methods. The antistreptolysin O, or ASO, titration is commonly carried out, but may require caution in interpretation in that human serum may in some conditions, especially hepatitis, have an inhibitory activity associated with lipid content and unrelated to antibody.

Quite aside from the question of the etiology of such diseases, the occurrence of such antibodies in the human population is not uncommon, as might well be expected from the frequency with which streptococcal infection occurs. It is sometimes stated that 70 to 80 per cent of persons having streptococcal disease show significant increases in antibody titer to streptolysin O, streptokinase, and hyaluronidase. The erythrogenic toxin also stimulates the formation of specific antitoxin, following both clinical scarlet fever and immunization with the toxin, and, like diphtheria antitoxin, the incidence of its occurrence rises with age, indicating that immunization occurs without the intervention of clinical scarlet fever. Intradermal inoculation of erythrogenic toxin gives a skin test, analogous to the Schick test in diphtheria, which allows the estimation of the immunity of the individual to the toxin. Antibody to the erythrogenic toxin cannot be titrated by in vitro methods, or to a satisfactory degree in experimental animals because of their relative lack of susceptibility to its action.

Differentiation must be made between an immune response in the technical sense of antibody formation and an effective immunity which prevents and/or modifies the infection or disease. The only immunity to streptococcal disease that is unquestionably effective is immunity to the erythrogenic toxin, which is reflected as immunity to the clinical disease scarlet fever. In general, immunity to streptococcal infections is of a low order and in any case transient. That some degree of effective immunity to a given type of streptococcus may be produced is indicated by recovery from and elimination of the infection in naturally occurring disease, and there is also some experimental evidence of the existence of an effective immunity. It has been found, 106 for instance, that a nasopharyngeal carrier state induced in monkeys resulted in an increase in antistreptolysin O titer of the serum and a resistance to reimplantation with the same strain that persisted for some months.

Unfortunately such immunity appears to be type-specific and, as pointed out earlier, associated with antibody to the M antigen. and there is only a small degree of crossimmunity. Since the M antigen appears to be located primarily, or even exclusively, in the cell wall, it has been inferred by some that its association with virulence is analogous to that of the pneumococcus capsule in that it interferes with phagocytosis. It would follow, then, that the effective immune response is opsonic, and studies utilizing the phagocytic index and similar criteria show a correlation between effective immunity and this humoral antibody. With the multiplicity of streptococcus types in the species Str. pyogenes, an effective immunity to streptococcus infection appears to be a somewhat impractical end.

Chemotherapy. The chemotherapeutic efficacy of the sulfonamides was first observed in experimental infections with Str. pyogenes. These compounds continue to be effective chemotherapeutic agents in infections with the streptococci but have been displaced to a considerable extent by the antibiotics, especially penicillin. Penicillin has been of special interest in the mass prophylaxis of epidemic streptococcal infection and in reducing the carrier rate in military populations.84 The hemolytic streptococci are more susceptible to these agents in vitro than are the staphylococci. The susceptibility of the green streptococci to the antibiotics is, in general, approximately the same as that of the staphylococci, but is subject to such wide variation from strain to strain that antibiotic sensitivity tests are practically mandatory. Bacterial endocarditis of α -hemolytic streptococcal etiology is the most important of the diseases produced by these streptococci, and the susceptibility of the causative strain is usually such that massive therapy is effective.⁵⁴

The relative efficacy of chemotherapy is dependent upon the kind of disease produced by the streptococci. For example, puerperal sepsis, streptococcal sore throat, pneumonia. and ervsipelas generally respond well to chemotherapy, but the rash of scarlet fever is not affected, nor is the therapy of acute rheumatic fever satisfactory. Chemotherapy does eliminate the focus of infection in scarlet fever and decreases the incidence of suppurative complications. The failure of rheumatic fever to respond to antimicrobial therapy is due to the lag of symptoms behind the actual infection and probable role of hypersensitivity (see below). Prevention of recurrent streptococcal infection in affected individuals is highly significant, and repository penicillin is an effective prophylactic agent.94

As in certain other infections, the prompt use of effective chemotherapeutic agents, especially penicillin in streptococcal infections, inhibits the immune response as compared to the untreated infection, probably as a consequence of reduction in the total

antigenic stimulus.

Bacteriological diagnosis of streptococcal infection. 111 Isolation of streptotococci from specimens of pathological material is ordinarily not difficult. An enriched medium is required, and blood agar is the medium of choice, for both α -hemolysis and β -hemolysis are apparent. Overgrowth by Proteus in cultures of some kinds of specimens may be prevented by including 0.02 per cent sodium azide in the medium. Most specimens, such as throat swabs and pus, may be streaked directly on blood agar, but enrichment culture, as in veal infusion broth containing 0.1 per cent dextrose and 0.1 per cent phosphate buffer, should be made with blood taken for culture, incubated for 24 hours, and then streaked on blood agar. If there is reason to believe that the specimen contains sulfa drug, its bacteriostatic effect may be neutralized by including 5 mg. per 100 ml. of p-aminobenzoic acid in the med-

The colonial morphology is typical in the case of β -hemolytic streptococci, and the characteristic chains of cocci may be found in Gram-stained smears. Green streptococci from sputum and similar specimens must be differentiated from pneumococci by inulin fermentation and bile solubility. The hemolytic streptococci may be typed by agglutination with type-specific antiserums and by

the precipitin test. For the latter the sedimented bacteria from a 250 ml. broth culture are suspended in 10 ml. of M/10 HCl in saline, boiled for 10 minutes, cooled in running water, and the insoluble material spun out to leave a clear supernatant to be used as the antigen. Considerable economy of reagents may be effected by setting up the precipitin test as a ring test in capillary pipettes; the precipitate at the serum-antigen interface may be observed with a hand lens.

There has been some interest in the use of the fluorescent antibody technique for the identification of Group A streptococci,⁴¹ but the method has been considered⁷¹ of limited value as applied to direct throat smears.

STREPTOCOCCAL INFECTION OF THE SKIN AND SUBCUTANEOUS TISSUES

Erysipelas. The ability of streptococci to infect the skin and adjacent tissues is well illustrated in erysipelas, an inflammatory disease of the skin caused by Str. pyogenes. There is some evidence that an attack of the disease is preceded by streptococcal infection of the throat or elsewhere in the upper respiratory tract, and it has been found that some individuals at least have the same immunological type of streptococcus in the throat as in the skin lesions. It is not clear whether the skin is directly invaded. or whether the microorganisms reach the area by some internal route, but the latter is only suggested and by no means established. The etiological relationship of Str. pyogenes to the disease is indicated by its presence, frequently in enormous numbers, in the lesions, the production of erysipelaslike disease in rabbits by the inoculation of streptococci, and inoculation experiments in carcinomatous patients which have demonstrated that pure cultures of streptococci can provoke the erysipelatous process. It is possible that hypersensitivity may contribute to the pathogenesis of the disease.3

Streptococci are not present in the central portion of the inflamed area, but are found on its periphery, and can be isolated most readily by excision of portions of the tissue, other methods rarely succeeding. In the skin they occur chiefly in the lymph

spaces, which are often packed with them, and may be recovered by skin puncture as far as 3 cm. beyond the advancing edge of the lesion where there is no gross evidence of inflammation. The hypothesis that the inflammatory reaction is due in part at least to the erythrogenic toxin has been an attractive one, but it seems definitely established that there is no relation; immunization with erythrogenic toxin, for instance, in no way prevents or reduces the inflammatory reaction in erysipelas.

This disease and especially experimental erysipelas in the rabbit, has been of considerable interest in connection with problems of local and tissue immunity. It is well established that one attack of the disease confers no protection against subsequent attacks, and in the opinion of many, some persons have a predisposition to the disease, suffering repeated attacks throughout life and even in the same areas. In the experimental disease in the rabbit, however, a number of workers have reported that successive intradermal inoculations bring about an increased resistance to subsequent inoculation in that area, and that with continued immunization the area increases slowly in size. The immunity is not highly specific, and inoculation of sterile broth also results in some increase in resistance to infection. Consistent with this, immune serum, when mixed with streptococci and inoculated intradermally, seems to have some protective effect. Serum therapy of the human disease, however, appears to produce only a possible mildly favorable effect upon the immediate attack but none upon recurrences and complications such as abscess formation.

Wound infection. 67 The green streptococci seldom occur in infections of wounds, but Str. pyogenes produces a suppurative infection when present, especially late. This organism is not normally present on the skin, and, in fact, normal skin has a bactericidal effect upon it. The relative rarity of infection of wounds by these bacteria is consistent with this, and streptococcal infection in most instances is a result of subsequent contamination by direct contact rather than of a primary infection. Str. pyogenes may occur alone or in mixed infections with other pyogenic bacteria such as staphylococci.

Cellulitis.⁵² Traumatic invasion of the skin and subcutaneous tissues may not remain localized but may develop into an

acute, spreading infection of the subcutaneous tissue with invasion of the muscle giving rise to gangrenous myositis. The infection to the subcutaneous tissues may show little or no evidence of localization and is characterized by the formation of a seropurulent exudate. It tends to spread rapidly via the lymphatic tissues and generalize into septicemia. This kind of streptococcal infection has been termed cellulitis, and may result from *Str. pyogenes* alone or, in the development of the gangrenous process, more often in mixed infection with anaerobic streptococci. This kind of wound infection was found in World War II.

STREPTOCOCCAL INFECTION OF THE UPPER RESPIRATORY TRACT¹⁰

As indicated earlier, the β -hemolytic streptococci occur most commonly as parasites and pathogens of the upper respiratory tract. By far the largest proportion of human disease caused by Str. pyogenes results from infection of the upper respiratory tract and adjacent areas, symptoms arising not only from the acute infectious process, but also in connection with its complications. The clinical character of the disease is determined by the relative prominence of the various results of the infection and, while seemingly different, is fundamentally the same. Thus streptococcal pharyngitis or septic sore throat becomes scarlet fever when the infecting strain of Str. pyogenes produces erythrogenic toxin, the infection commonly extends into the tonsils or may be localized primarily there to give clinical tonsillitis; it may extend into the sinuses or middle ear to produce streptococcal sinusitis and otitis media respectively, and by extension into the lungs result in bronchopneumonia of streptococcal etiology. Further, late nonsuppurative complications of streptococcal infection include carditis, nephritis, and arthritis. While separation of B-hemolytic streptococcal infection into various clinical entities has some practical value, the basic infectious process is essentially the same.

The green streptococci are also inhabitants of the upper respiratory tract, as pointed out earlier, and are so constantly present and of such restricted pathogenicity that they are a part of the normal bacterial

flora. Infection of the teeth and gums undoubtedly stems from this area, and it is likely that the bacteria entering the blood stream to produce local infections elsewhere in the body originated in the upper respiratory tract. They are occasionally found in bronchopneumonia. 92, 97

Streptococcal sore throat (septic sore throat). The β -hemolytic streptococci are responsible for an acute infection of the throat commonly known as septic or streptococcal sore throat. Epidemics of this disease appeared in the United States and in England during the first decade of the present century. The immediate symptoms in these and subsequent epidemics have been strikingly similar and include an intense local hyperemia, with or without a gravish exudate, enlargement of the cervical lymph nodes, and usually fever. Extension of the infection into the lungs may occur with resulting streptococcal pneumonia³⁵ which may terminate in fatal septicemia, and peritonitis has been a cause of death also. The disease, usually in a relatively mild form, is a common one.

The sequelae of streptococcal sore throat include those resulting from the extension of the infection into adjacent areas such as the sinuses and middle ear, and purulent, semichronic infections often develop. Streptococci also frequently persist in tonsillar crypts in a chronic type of infection which may flare up periodically in an acute form. Streptococcal tonsillitis, sinusitis, and otitis media are, then, a part of the pathology of hemolytic streptococcal infection of the throat. In addition to such extensions, the effects of toxemia on other parts of the body are evident as carditis, nephritis, and arthritic involvement of the joints. As in the case of scarlatinal rash noted earlier, the character of an epidemic is often recorded as percentage incidence of these various sequelae.

The distinctive clinical character and epidemic spread of the disease led earlier workers to believe that it was caused by a particular kind of streptococcus to which the name Str. epidemicus was given. It is now established that various immunological types of Str. pyogenes are, for the most part, responsible for the disease, and a small portion of the cases are infections with streptococci of Group C, now grouped as Str. equisimilis. On the other hand, there is reason to believe that so-called "epidemic

strains," of high virulence and infectivity, of streptococci as well as other bacteria are often associated with epidemic disease.

It is probable that the infection is largely droplet and airborne, including dust, but there is no doubt that in many instances direct contact is of considerable significance. It may also be transmitted through food and milk and streptococcal infection is often milkborne. In the past it has been assumed that the contamination was direct from man, but evidence has accumulated which indicates that mastitis of *Str. pyogenes* etiology may constitute the immediate source of infection of the milk.

SCARLET FEVER

Scarlet fever is a clinical entity because of the rash resulting from the action of the erythrogenic toxin; otherwise it does not differ significantly from other streptococcal infection of the upper respiratory tract, and its sequelae are essentially the same.

While scarlet fever has declined in prevalence and severity since the turn of the century, following a period of high prevalence during the nineteenth century, it has behaved differently in different parts of the world in the last two or three decades. It increased in continental Europe after World War II and remained at a high prevalence, while in England and Wales it continued at a fairly constant level, and has declined sharply in the same period in the United States and Canada.⁶⁴

The relationship of β -hemolytic streptococci to the disease was demonstrated by Dick and Dick in 1923 by the reproduction of typical scarlet fever in human volunteers, by the inoculation of pure cultures, and by the demonstration of the existence of the erythrogenic toxin. A conclusive demonstration of the etiological relation was required because of the contrast between the relatively lasting immunity to scarlet fever following recovery from an attack of the disease and the transient immunity to other streptococcal infections. It has been held by the Dicks and others, largely on the basis of early agglutination studies, that the scarlet fever streptococci constitute a homogeneous group of \(\beta\)-hemolytic streptococci which should be designated Str. scarlatinge. While the streptococci found in scarlet fever are all members of Group A, i.e., Str. pyogenes,

the ability to form erythrogenic toxin is not confined to any particular type within this group, though some types occur more frequently than others, and it cannot be said that the scarlet fever streptococci are immunologically homogeneous. Neither are they biochemically homogeneous, and in the Dicks' early experiments the mannitol fermentation was variable in scarlet fever-producing strains. It appears, then, that the scarlet fever streptococci are strains of *Str. pyogenes* that have in common the ability to form erythrogenic toxin, but such strains are found in conditions other than scarlet fever, such as erysipelas.

The erythrogenic toxin.¹⁷ It is generally agreed that the scarlatinal toxin is protein in nature, but some workers have reported that it is heat-stable and others that it is heat-labile. It has been found, however, that heat-lability is determined by methods of preparation. The purest preparations, prepared by ammonium sulfate and cold ethanol fractionation, contain 11,250 Lf/mg. N or 2100 Lf/mg. protein. Four components have been demonstrated by electrophoresis; the toxicity was associated with the slowest, and on separation this contained 108 STD per mg. Whatever its nature, it is necessarily assayed by skin test and its potency is measured in skin test doses (STD), the smallest amount that will produce the characteristic erythematous response. It is antigenic and is flocculated by antitoxic serums but in multiple zones, and the flocculation titration must be carefully controlled.36

Scarlet fever antitoxin. The therapeutic use of antistreptococcus serums was investigated by Moser in 1902 with encouraging results, and similar observations were reported by Dochez and others in 1924. The Dicks prepared specific antitoxic serums by immunizing horses with sterile culture filtrates. The results of the therapeutic use of the Dick antitoxin are favorable on the whole, though the mildness of the prevalent type of scarlet fever makes it difficult to secure any large body of statistics as cogent as those recorded in diphtheria. In individual cases, however, the administration of antitoxin decreases the duration of the rash, changes the character and extent of desquamation, and reduces the number of complications, and there is general agreement as to the efficacy of antitoxic serum properly prepared and administered early. Some clinicians would restrict the use of scarlet fever antitoxin to cases of severe or toxemic type. A unit of antitoxin is defined as that amount which will neutralize 50 skin test doses of toxin.⁷⁰

Immunity to scarlet fever is, in a sense, a clinical immunity in that it is largely an immunity to the erythrogenic toxin rather than to the streptococcus. It may be demonstrated by the Dick test, a skin test analogous to the Schick test in diphtheria; i.e., the local erythema is due to the action of the toxin and is absent in the presence of antitoxin, either of exogenous origin or present in the immune individual. The Dick test may be used, then, to ascertain whether or not an individual is immune to scarlet fever or. more precisely, whether circulating antitoxin is present. In this connection it is of interest that Schultz and Charlton earlier observed that when a scarlet fever patient with a bright-red rash is injected with 1 ml. of convalescent serum, after about six hours the rash begins to fade and soon disappears completely. The significance of this phenomenon, the Schultz-Charlton blanching phenomenon, was not recognized at the time.

Scarlet fever, like the other hemolytic streptococcal infections, may be effectively treated with penicillin, to reduce the period of illness and the number of complications. Early chemotherapy seems to interfere with the development of the immune response, as indicated by the antistreptolysin titers, and the relapse rate is not reduced.⁸⁵

Prophylactic inoculation. The It is not generally known that following Jenner's work on smallpox vaccination attempts were made to immunize against scarlet fever by a similar process of inoculation. Preventive inoculation with killed streptococcus cultures was practiced by Russian bacteriologists as early as 1906. Mild symptoms were produced similar to those observed in scarlet fever. A single injection did not suffice to produce immunity, two or three inoculations being necessary. It was believed that a considerable degree of immunity was obtained by this procedure.

The discovery of the scarlet fever toxin offered an opportunity for protective immunization similar to that successfully utilized to diphtheria. Toxins with a potency of at least 40,000 STD per cubic centimeter are preferable, and appropriate dilutions are injected at intervals of one week. Five injections are recommended, starting with 500 STD and gradually rising to about

100,000 STD. Immunization of susceptibles (Dick-positives) in this manner produces 98 per cent or more Dick negatives. The injections are sometimes accompanied by the development of a scarlatiniform rash and other symptoms of mild scarlet fever. In consequence, the use of toxin detoxified by treatment with formalin has been advocated by some workers. It is to be emphasized that immunity to scarlet fever is an immunity to clinical scarlet fever, not to the streptococcal infection, and from the epidemiological point of view both Dick-negative and Dick-positive individuals must be considered as having scarlatinal infections. the only difference being the clinical one dependent upon the development of a rash.

As in the case of diphtheria, immunity to scarlet fever may be acquired by inapparent infection. The frequency of positive Dick tests is low in newborn children (indicating a passive immunity of maternal origin), then rises to a maximum in the age groups one to five years, and thereafter falls off gradually until in persons over 30 it is relatively low, possibly 15 per cent.

RHEUMATIC FEVER 42, 91, 99

As indicated earlier, not uncommon sequelae of β -hemolytic streptococcal infection include carditis and arthritic involvement of the joints. These are emphasized and assume a major role in the pathology and symptomatology of the post-streptococcal nonsuppurative inflammatory disease known as rheumatic fever, acute rheumatism, or rheumatic heart disease and occurs as a sequel to streptococcal infection in about 3 per cent of cases in closed populations, but at a lower rate, 0.1 to 0.3 per cent, in the general population.

The essential lesion of rheumatic fever is carditis, which may or may not be accompanied by pyrexia and arthritis. The carditis includes the connective tissue degeneration characteristic of the damaged heart valves, and specific inflammatory myocardial lesions characterized histologically by nodular collections of cells described by Aschoff and known as Aschoff nodules. Rheumatic fever is the third most common infectious disease in this country, exceeded only by tuberculosis and syphilis.

The connection between β -hemolytic streptococcal infection and rheumatic fever

is very close, and there is good reason to believe, though as yet not complete and unequivocal proof, that the relationship is etiological. The epidemiological association of streptococcal infections such as tonsillitis, puerperal fever, scarlet fever, and the like, with rheumatic disease has long been recognized. It is reinforced by evidence of an immunological response, such as rising ASO titers, to streptococcal infection almost invariably associated with rheumatism; an ASO titer of 200 or more is generally considered to be indicative of recent infection.

Rheumatic carditis occurs in some individuals; the lesion is a nonpurulent inflammation of the valve cusps and rings and formation of verrucae on the margins which are eventually replaced with scar tissue. Rheumatic myocarditis commonly occurs also, and is characterized histologically by the perivascular granulomata known as Aschoff bodies or nodules. Dome observations suggest that there is an individual tendency to valvular involvement, and when this does not occur in the first attack, it is less likely to in subsequent attacks.

The onset of the disease does not necessarily coincide with acute streptococcal disease and only rarely has it been possible to culture β -hemolytic streptococci from the blood. Largely through the work of Coburn, beginning in 1931, these discrepancies have been reconciled, and the etiological relationships of the β -hemolytic streptococci clearly indicated.

Coburn differentiates three stages in the disease: first, an acute streptococcal infection of the nasopharynx; second, a period of quiescence during which streptococci remain the the throat; and third, the stage in which electrocardiographic changes are apparent and the symptoms of acute rheumatic fever appear. During the third stage streptococci may or may not be demonstrable, and the symptom complex appears to be a consequence of a developed hypersensitivity to the streptococcal antigens (see below). The time which elapses between the first and third stages varies from one to five weeks in patients in whom it has been studied. A practically significant point is that, since clinical rheumatic fever is not a direct result of streptococcal activity, chemotherapy is not effective.

Subsequent attacks occur on reinfection with streptococci, or occasionally following

nonspecific febrile events, and control of the disease in the individual consists in chemoprophylaxis. Repository penicillin, e.g., benzathine penicillin G, and sulfonamides are satisfactory, but the broadspectrum antibiotics are not. The time over which prophylaxis must be continued is uncertain, but some evidence suggests that recurrences drop sharply after age 18.38

The mechanism by which streptococci produce rheumatic fever is far from clear. The antibody response to the initial infection is delayed in that peak titers of antistreptolysin O, complement-fixing antibody, and group- and type-specific precipitins occur in the third stage of the disease rather than earlier, while antistreptolysin S declines in the third stage, particularly in those having recrudescences. There is reason to believe that hypersensitivity exists to the streptococcus or its products; for instance, joint pains are often produced in rheumatic patients by the inoculation of sterile streptococcus culture filtrates. It appears that an immune response is basic to these consequences of streptococcal infection, but whether this is a conventional antibody response or a hypersensitivity, or both, is not clear. As regards the former, there is reason to suspect that an auto-immune response occurs³⁹ which would put rheumatic fever and its sequelae with the collagen diseases; there is, for example, evidence of bound y-globulin in cardiac tissue. A sensitization may be one to streptococcal antigen, and if so it is an antigenicity widely distributed over types of Str. pyogenes, or to a complex of streptococcal and tissue antigenicities.40

It is quite likely also that the role of the host is of considerable importance. There is evidence of hereditary susceptibility to rheumatic fever, 115 and some indication of a constitutional predisposition to rheumatic fever; the disease occurs more commonly in hyperthyroid persons, there is some evidence suggesting that lipid metabolism may be involved, and it occurs more commonly in the 5- to 15-year age group than in older persons.

It has been of some interest that many of the lesions characteristic of rheumatic fever may be produced in the rabbit⁶² by inoculation of β -hemolytic streptococci, and with cell-free sonic lysates of streptococci in the mouse.¹³ These include nonsuppurative

myocarditis (without, however, the typical Aschoff nodule), endocarditis, and arthritis ranging from mild to severe with extensive changes in the bone. While it may be argued that such pathology is not identical in all respects with that of rheumatic fever in man, the fact that such lesions can be produced experimentally by the inoculation of streptococci or their products may be regarded as strong evidence in support of the assumption that these bacteria may be etiologically related to human rheumatic fever.

In general, then, it seems quite clear that there is a very close, probably etiological, relation between *Str. pyogenes* and rheumatic fever, but the pathogenesis of the disease is far from clear.

ACUTE GLOMERULONEPHRITIS

An association of acute hemorrhagic glomerulonephritis with streptococcal infection has been known for many years, beginning, in fact, with Bright's description of the condition in association with scarlet fever as early as 1836. A causal relation between streptococcal infection and this disease appears to be definitely indicated by application of typing methods to give a high correlation between this kind of nephritis and certain types of streptococci.^{72, 108, 113}

In contrast to rheumatic fever, which occurs as a complication of Str. pyogenes infection with all serological types at a more or less constant rate of around 3 per cent, the attack rate in glomerulonephritis associated with streptococcal infection is highly variable, and extremes of 0.03 and 18 per cent have been observed. The disease may occur in semi-epidemic form, especially in school children. This may be taken to suggest that, unlike rheumatic fever, glomerulonephritis may reflect the variable distribution in time and space of nephritogenic strains of Str. pyogenes. 69 In apparent confirmation of this, it has been found that the majority of cases in this country which have been studied bacteriologically have been associated predominantly with Str. pyogenes type 12, a lesser number with type 4, and occasionally other serological types may be implicated. An apparently new type of Str. pyogenes, the Red Lake strain. occurring in an epidemic of nephritis on an Indian reservation has been described

Fred Helmer Care Control

also. 100 A similar association with type 12 has been observed also in Canada and in England. It is by no means certain that all type 12 strains are nephritogenic, and strains apparently differ among themselves in this respect. Other than this association with serotype, nephritogenic strains of *Str. pyogenes* are not differentiable from those which are not associated with kidney disease.

Glomerulonephritis differs from rheumatic fever also in that it does not tend to recur in the recovered individual, and it is supposed that an effective degree of immunity to the type-specific M antigen persists and that a hypersensitivity is not involved in the disease. Recovery usually seems to be complete, and there seems to be no evidence that chronic nephritis is causally related to prior streptococcal infection.

The disease has been reproduced in rabbits⁷³ and monkeys⁷⁴ by infection with nephritogenic type 12 strains of *Str. pyogenes* or with products of the bacteria administered repeatedly, and in mice with soluble streptococcal products in *in vivo* diffusion chambers.^{44, 96} Renal lesions have also been produced in experimental animals by inoculation of soluble antigen-antibody complexes^{60, 103} and in parabiosed animals.⁵⁰ The evidence shows that kidney pathology results from the antigen-antibody reactions following immunization to streptococcal antigen, and are an indirect, rather than a direct, consequence of the primary infection.

SUBACUTE BACTERIAL ENDOCARDITIS

Infection of the endocardium with the formation of ulcerative lesions may occur with many bacteria. 101 By far the most common are the α -hemolytic streptococci, but staphylococci, \(\beta\)-hemolytic streptococci, and, to a much lesser extent, pneumococci, gonococci. meningococci. Hemophilus, Brucella, Salmonella, etc., are found. With the more highly virulent bacteria the infection is an acute one, but the disease known as subacute bacterial endocarditis, which is no less fatal, is caused almost exclusively by an α -hemolytic streptococci, though in a very few instances anhemolytic streptococci have been implicated.

The infection of the heart valves may be

primary or secondary to a focus of infection elsewhere in the body, and an almost indispensable predisposing factor is congenital abnormality or prior damage, as by rheumatic infection, of the heart valves. The source of infection is often the tonsils and periapical infections of the teeth or lowgrade infection in the gums. There seems to be an occasional leakage of streptococci from these sources into the blood stream. and any disturbance of the infected areas, such as the extraction of teeth, tonsillectomy, and manipulation of an infected cervix. results in transient bacteremia. The bacteria are rapidly removed from the blood stream by phagocytic cells in the liver, spleen, bone marrow, and elsewhere in the body, but infection of the heart valves may occur when there is an abnormality. Such abnormality may, for example, be a consequence of cardiac damage occurring during an attack of rheumatic fever.

The infection of the endocardium is probably direct rather than from embolism in the smaller vessels at the attachment of the endocardial and subendocardial tissues. According to Grant, Wood, and Jones²⁶ the bacteria establish in small platelet thrombi on the valvular surfaces, but McNeal, Spence, and Slavkin⁵⁸ found that, in experimental endocarditis in the rabbit, the circulating bacteria are phagocytosed by the endothelial cells on the heart valves as well as elsewhere, but are not killed and produce local damage which is covered by a deposit of fibrin; the fibrin protects the streptococci to allow proliferation and the development of a local infection. In any case, during the course of the disease streptococci are shed into the blood stream and may be demonstrated by blood culture. It is significant, especially from the point of view of chemotherapy, that the lesion is not a superficially situated thrombus, but is, for all practical purposes, an abscess in the substance of the valve.

Species of green streptococci of both the viridans and enterococcus groups are responsible for this disease. In one study it was found that practically all of nearly 200 cultures of green streptococci from clinical sources, including subacute bacterial endocarditis, were either Str. salivarius or Str. faecalis; and in another 12 the most common species found was Str. mitis, Str. salivarius next, and Str. faecalis was found least often.

The disease process is, however, essentially the same regardless of the species of α -hemolytic streptococcus causing it.

PUERPERAL FEVER

Puerperal fever is a vague term in that some degree of pyrexia is not uncommon immediately following childbirth. A definite febrile response is probably in most instances associated with bacterial infection, but in mild cases the microorganisms are relatively avirulent. The severe infections are due almost entirely to streptococci, the majority Str. pyogenes. Infection with other β -hemolytic streptococci occurs, but when these are of groups other than Group A the infections are usually not severe. The anaerobic streptococci are second only to Str. pyogenes in importance, being the cause of possibly 20 to 25 per cent of cases of severe puerperal fever; the disease caused by these forms is not as rapidly fulminating as that caused by Str. pyogenes, but the case fatality rate is high, possibly 40 per cent. In fatal cases of puerperal fever the infection generalizes to fatal septicemia, and it is probable that the pronounced invasive qualities of the streptococci are responsible for their virulence under these circumstances.

A point that has been of primary interest is the source of the streptococci producing puerperal fever. It seems well established that Str. pyogenes occurs only rarely in the female genital tract and is rarely found before labor or during an afebrile puerperium. Lancefield and Hare,49 for example, reported a series of 855 cultures of the vagina of parturient women; one strain of Str. pyogenes was found among 65 strains isolated during an afebrile puerperium, and none among 13 strains isolated before labor. This and similar studies indicate that the source of infection with Str. pyogenes is exogenous rather than endogenous. The development of streptococcus typing and a reasonably precise definition of the immunological types has allowed the demonstration of the probable source of the infecting organisms. In one study, slightly over 50 per cent of the streptococci from the patient were identical with those from the nose and throat of attendants. and a little less than 25 per cent identical with those from the nose and throat of the patient. Colebrook⁹ reported similar results, the percentages being 58 and 38 respectively. Data such as these show that the source of infection is exogenous with respect to the genital tract and most probably the nose and throat of attending persons or the patient herself in 75 per cent or more of cases. The infection may be air- or dustborne, but it seems probable that the hands play a significant part in the transfer of infection.

The anaerobic streptococci, on the other hand, are normal inhabitants of the human vagina.⁹⁵ In the absence of precise immunological methods for their identification, it may be tentatively concluded that infection with these organisms to produce puerperal fever is endogenous for the most part.

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Chapter Eighteen

THE PNEUMOCOCCI®

The bacterium most commonly found in pneumonia in man is a small lanceolate micrococcus which has been variously termed Micrococcus pneumoniae, M. lanceolatus, Streptococcus pneumoniae or, more briefly, the pneumococcus or Fränkel's pneumococcus. Diplococcus pneumoniae is now the commonly accepted formal name.

Of the generally recognized anatomical types of pneumonia-lobar or acute croupous pneumonia, bronchopneumonia or lobular pneumonia, and capillary bronchitis or bronchiolitis-lobar pneumonia is nearly always due to the pneumococcus, though other bacteria are occasionally involved. Perhaps the best quantitative data are those assembled by Rumreich and his co-workers⁴³ in a two-year study in six states representing high and low pneumonia rates. These are summarized in the accompanying table. It is evident that the pneumococcus is by far the commonest cause of pneumonia. The microorganisms associated with bronchopneumonia are varied, and their source is probably almost always the nasopharynx.45

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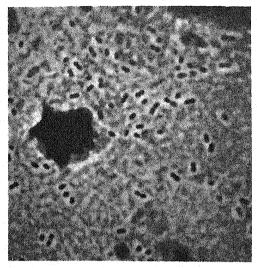
The pneumococcus was discovered independently in France in 1881 by Pasteur, who inoculated rabbits with the saliva of a child dead of rabies, and by Sternberg in the United States, but was not known to be associated with disease in man before the extensive investigations of Fränkel and Weichselbaum, who demonstrated conclusively the etiological relation of this bacterium to pneumonia in man.

Morphology and staining. The pneumococcus is typically a small, slightly elongated coccus, one end of which is pointed or lance-shaped. The cocci commonly occur in pairs (diplococci), but variations both in grouping and in size and form of individual cells are frequently observed. Chain formation is common, especially in artificial mediums, although the chains are usually shorter than those of *Str. pyogenes*. Oval and elongated bacillary forms sometimes occur. The pneumococcus is nonmotile and does not form spores. A well-defined capsule envelops the pneumococci in animal exudates but, except in certain strains or in certain

The Incidence of Etiological Agents in Pneumonia*

CAUSATIVE ORGANISM	LOBAR PNEUMONIA	BRONCHO- PNEUMONIA	UNSPECIFIED	ALL PNEUMONIAS
Pneumococcus	82.48	65.79	77.48	77.71
Hemolytic streptococcus	2.00	3.33	3.99	2.65
Other streptococci	1.30	2,99	1.33	1,70
Staphylococcus	0.82	2.00	1.38	1.19
Friedländer's bacillus	0.15	0.13	0,28	0.17
Influenza bacillus	0.06	0.25	0.11	0.15
Tubercle bacillus	*****	0.08		0.02
Fungi	,,,,,,	0.02	*****	******
Virus	*****		0.07	0.01
No significant organism recorded	13.19	25.41	15.38	16.44
Number of cases	15,420	6,092	4,290	B5,802

^{*}In six representative states over a two-year period as compiled by Rumreich et al.43



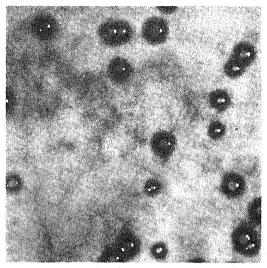


Figure 88. Left, pneumococcus in the peritoneal fluid of a mouse. Note the capsules. Fuchsin; \times 2200. Right, Colonies of the pneumococcus on blood agar. The areas of green hemolysis have been accentuated in the photograph. \times 3.

mediums, is less readily demonstrable in cultures grown outside the body. Capsules may often be found in milk cultures and in mediums containing blood or serum.

The pneumococcus is readily stained with the aniline dyes and is generally gram-positive, although there is a tendency to become gram-negative in older cultures, and occasional strains are found to be gram-negative. In stained preparations the capsule may often be seen as an unstained halo surrounding the cells; it may be stained by special methods.

The colonies of pneumococcus on infusion agar or blood agar are typically small, moist, translucent, and granular, with well-defined edges. These bacteria are α -hemolytic on blood agar, and the colonies appear surrounded by a zone of greenish discoloration on this medium and resemble the colonies of green-producing streptococci, so closely as to be practically indistinguishable from them.

Physiology. Some pneumococci grow upon the ordinary nutrient (beef extract) culture mediums but many do not,²⁵ and, in any case, growth is sparse. Nutritional requirements are complex. A number of semi-synthetic mediums have been developed, based on gelatin or casein hydrolysate, and supplemented with vitamins and various other growth accessory substances, and amino acids.^{1, 8, 9, 19, 40, 41} The requirement for added choline, nicotinic acid, and pantothenic acid appears to be quite general

among the serotypes, and biotin and ascorbic acid are required by some strains. Growth is often markedly stimulated by the inclusion of purines and pyrimidines, but these do not appear to be essential. Amino acid requirements are complex and varied from strain to strain. Of the common serotypes, type 3 appears to be more exacting, but strain differences may be as great, or greater, than differences between serotypes.

Growth on infusion mediums, particularly those enriched by the addition of whole blood, takes place at 37° C. Litmus milk is promptly acidified and often, but not invariably, coagulated. The temperature range over which these bacteria may be grown is relatively narrow (25° to 42° C.), and they are sensitive to variations in pH from the optimum of 7.8, the limiting acidity and alkalinity being 6.5 and 8.3 respectively. The pneumococcus is a facultative anaerobe, although certain other species of Diplococcus are obligate anaerobes.

In general, sugars are actively fermented with the production of large amounts of lactic acid and small amounts of volatile acid and ethyl alcohol. Differential fermentations are of no particular value in the classification of these microorganisms except in the case of inulin, which serves to differentiate the pneumococci and the green streptococci.

The pneumococci appear to be structurally delicate organisms and autolyze much more readily than most other kinds

TOXINS 455

of bacteria. Evidences of protein hydrolysis accompany autolysis, and it appears that lysis is a consequence of the activity of intracellular enzymes.39 Perhaps associated with the autolytic process is the lysis of pneumococci by bile and bile salts. The socalled bile solubility of pneumococci is practically a constant characteristic, although different strains vary in their sensitivity to bile as they do in the tendency to autolysis. Rabbit or ox bile may be used and added to a young broth culture in the proportion of 10 to 20 per cent. Solutions of pure bile salt (sodium taurocholate) are preferable to ox bile because they may be sterilized and the concentration (10 per cent) controlled. Heat-killed pneumococci are not bile-soluble. Sodium lauryl sulfate and similar detergents will also lyse pneumococci. 10, 20

Pneumococci may, therefore, be distinguished from streptococci by their bilesolubility and, less definitely, their ability to ferment inulin, in addition to their greater pathogenicity for mice and the characteristics of the colonies on agar.

Peroxide is found in considerable quantities in pneumococcus cultures after prolonged incubation because of the lack of catalase in these microorganisms. This, coupled with their sensitivity to peroxide, results in the autosterilization of cultures kept in the incubator for many days. Cultures in blood broth, however, remain viable for several weeks in the refrigerator, and the bacteria may be preserved for months in the cold in vacuum-desiccated spleens of infected mice.

The pneumococci are more sensitive to the bactericidal activity of the usual antiseptics than are many other bacteria. Soaps, such as ricinoleate and oleate, are pneumococcidal in relatively high dilutions, 0.04 and 0.004 per cent respectively, and other substances such as phenol and mercuric chloride are also highly effective in the destruction of these bacteria. Quinine and some of its derivatives have antipneumococcal activity but do not inhibit α -hemolytic streptococci; optochin-containing discs, similar to antibiotic discs, are available for the differentiation of pneumococcus and green streptococci.

Toxins. The severe intoxication observed in pneumococcal infection in man is suggestive of the formation of some toxin by this

bacterium. The existence of such a toxin has never been demonstrated, however, and the pneumococcus does not produce a toxin analogous to those of the diphtheria and tetanus bacilli.

Other toxic substances are produced by this microorganism. That there is α -hemolysis on blood plates has already been noted. and there is in addition a filterable hemolysin active on sheep, guinea pig, and human erythrocytes.35 The concentrated hemolysin is reported to have lethal and dermotoxic properties. The pneumococcus also produces a leucocidin³⁷ and a necrotizing substance similar to that formed by some of the staphylococci. Many strains produce hyaluronidase, especially when cultivated in mediums containing hyaluronic acid.27 A purpura-producing substance which is nonantigenic and appears to be a cleavage product of pneumococcal protein has been described by a number of workers. Injected into white mice, extracts of pneumococci produce a purpuric condition manifested as a dark blue discoloration of the skin of the feet, tail, ears, nose, and genitals.28 It has been observed too that virulent strains are able to utilize extracellular breakdown products of nucleic acids, which stimulate both respiration rate and DNA synthesis. 17

Although preparations containing these activities have been reported to increase the virulence of relatively avirulent pneumococcus strains when injected simultaneously with the bacteria, the virulence of pneumococci is directly dependent, not on the formation of such toxic substances, but on the production of specific soluble substance and encapsulation.

Classification. The pneumococci are closely related to the streptococci, but the degree of intimacy of the relationship is as yet open to question. Some workers regard these microorganisms as but a species of streptococcus and designate them as Str. pneumoniae. In general, however, it is customary to consider the pneumococci as a distinct genus, a practice which is justified by considerations of the sum of the characteristics of the pneumococci and the clinical and epidemiological aspects of the pneumonias. According to Bergey's classification, the tribe Streptococceae is made up of three genera: (1) Diplococcus, of which the type species is D. pneumoniae or the pneumococcus; (2) Streptococcus, with Str. pyogenes as the type species; and (3) Leuconostoc, a group of gas-forming, chainproducing cocci found in milk, fermenting vegetables, and slimy sugar solutions.

The genus Diplococcus includes six species in all, the five in addition to the pneumococcus being obligate anaerobes. *D. paleopneumoniae* closely resembles the pneumococcus except for its anaerobic character, occurs normally in the buccalpharyngeal cavity, and is reported to be highly pathogenic. *D. plagarumbelli* has been found in septic wounds, and the remainder, *D. magnus*, *D. constellatus*, and *D. morbillorum*, appear to be normal inhabitants of the mouth and intestinal tract and have been found in lymphoid tissue such as in the tonsils and appendix.

Pneumococcus types. A point that has been of somewhat more interest than the formal taxonomic position of the pneumococci is the subdivision of these bacteria into types. The pneumococci contain two kinds to antigen. One, the so-called somatic antigen, is a constituent of the cell substance proper and is immunologically identical in all pneumococci. The other, the polysaccharide haptene or specific soluble substance (SSS), is type-specific and serves to differentiate the immunological types of pneumococci from one another. The polysaccharide of type 3 has been the most thoroughly studied chemically and types 1, 2, 5 and 6 to a lesser extent (Chap. Three).23, 29 These substances have been isolated from most of the other types also. The presence of SSS masks the somatic antigen, and antiserums to encapsulated pneumococci are sharply type-specific. These immunological types are designated by numerals, and the numbering of the types, although purely arbitrary, is a generally accepted convention.

Observations by Dochez and Gillespie in 1913 showed that the pneumococci found in cases of lobar pneumonia can be divided into four distinct groups, designated as types 1, 2, and 3 and Group IV, on the basis of specific agglutination and protection tests. Of these, types 1, 2, and 3, found in the majority of cases of pneumonia and producing the most severe types of infection, have specific immunological character, while Group IV is immunologically heterogeneous and is made up of all pneumococci not belonging to the first three types.

The types making up Group IV were then sorted out by Cooper and her associates

in this country, and by Danish workers, to give a great many serological types of pneumococcus. There are two different nomenclatures for the pneumococcal types:30,31 the Danish system which is an extension of Cooper's system in which serologically related types are grouped together as subtypes, and the system prepared by Eddy¹² under the auspices of the U.S. Public Health Service and the American Public Health Association, in which the differentiable types are given consecutive numbers irrespective of antigenic relationships. There are 80 differentiable types, but some are antigenically related to one another; there is, for example, partial cross-reaction between types 2 and and 5, between 3 and 8, between 7 and 18, and between 15 and 30. Rarely, mixed types, containing antigens characteristic of more than one differentiable type, are found.18 Mixed types have been produced artificially by transformation reactions⁵ (see below), and such types are considered to contain two capsular genomes, one mutated and the other normal, which interact to give the observed phenotypic expression.6

Diblococcus mucosus. These nological types are culturally indistinguishable with the exception of type 3, which stands somewhat apart from the other pneumococci in that it produces a heavy mucoid growth because of its luxuriant capsule formation. It is considered by some to be a separate species, D. mucosus. Many cultures show a marked tendency to form chains, and the dividing line between D. mucosus and "Str. mucosus" is not a sharp one, if indeed any distinction should be made. These coccoid, heavily capsulated bacteria for the most part ferment inulin and are soluble in bile, so that the tendency is to group them with the pneumococci rather than the streptococi. A few mucoid strains have been reported which are bileinsoluble and noninulin-fermenting.

Pneumococcus typing. The serological typing of pneumococci may be carried out in a number of ways but is not a routine diagnostic procedure. The various methods that have been developed are basically immunological but differ in technical detail. The immunological procedures are of three kinds: (1) the agglutination of the pneumococci with type-specific antiserum, (2) the precipitation of SSS with type-specific antiserum, and (3) the Quellung reaction. The first two have been discussed in pre-

TYPES 457

vious sections and need not be considered further here.

The Ouellung phenomenon was described by Neufeld in 1902 and is in general use. A suspension of pneumococci is mixed with undiluted antiserum (rabbit serum is preferable to horse serum) on a slide or cover glass, a small amount of Löffler's alkaline methylene blue is added to facilitate observation, and the preparation is examined under the microscope. In the presence of homologous immune serum there is a marked apparent swelling of the capsule without any obvious change in the size of the bacterial cell itself; no such swelling is observed with heterologous serums. The reaction takes place rapidly, and the swelling is usually apparent within a few minutes. This apparent swelling of the capsule in the presence of specific antiserum is discussed elsewhere (Chap. Three).

The use of serum pools considerably facilitates typing, especially the identification of the higher types. The incidence of the various types determines the most advantageous combinations of antiserums. A group of combinations often recommended for use in this country is:

(a) 1, 2, 7

(b) 3, 4, 5, 6, 8

(c) 9, 12, 14, 15, 17

(d) 10, 11, 13, 20, 22, 24

(e) 16, 18, 19, 21, 28

(f) 23, 25, 27, 29, 31, 32

Monospecific antiserums are required also. The pneumococcus to be typed is tested with each pool and then with the component antiserums of the pool with which it reacts. Thus a pneumococcus may be identified in 12 or fewer tests. 13, 14, 15

Variation. The smooth and rough variants that have been found in a variety of bacteria may also be observed in the pneumococcus. As in other cases, there are various intermediate colonial types between the two extremes, and the pneumococcus is virulent in the smooth form and almost completely avirulent in the rough form. The change from smooth to rough is reflected in the microscopic morphology of the cells as a loss of capsule. According to Austrian, colonial type involves the formation of filaments of pneumococci as well as capsule formation; the typical virulent strain is the nonfilamentous capsulated

form. These factors vary independently, as shown by transformation reactions, to give the possible combinations, including a filamentous noncapsulated type which had not been explicitly recognized earlier.

Since type specificity is determined by the SSS, it follows that the change from smooth to rough is accompanied by a complete loss of type specificity; the somatic antigen is predominant and, irrespective of original type, the pneumococci become immunologically identical. The dissociative change may be reversed, although with difficulty, by animal passage or by cultivating the R form in the presence of anti-R immune serum or in the presence of heat-killed cells from a smooth culture.

Transformation of types. The experimental interconversion of pneumococcal types by direct transduction using an R form of pneumococcus and polymerized deoxyribonucleic acid prepared from a heterologous type has been described elsewhere (Chap. Seven) together with changes in serotype and other characteristics by phage-mediated transduction. These phenomena have been intensively studied with the pneumococcus, but the extent to which such changes may occur in nature is unknown.

Pathogenicity for man.21 As indicated above, lobar pneumonia is the most important pneumococcal infection in man. The bacteria are not confined to the lung, for they may migrate from this seat of infection through the nasal passages or be distributed via the vascular system to various parts of the body, to give rise to localized foci of infection. Pneumococcemia is of frequent occurrence; in one series of cases studied48 over a 14-year period, bacteremia averaged 22.8 per cent, the annual averages ranging from 16.4 to 49 per cent. A number of workers emphasize the prognostic value of blood culture, and in most instances both the case fatality rate and the incidence of purulent complications are considerably higher when pneumococci are present in the blood stream.7

Among the pathological processes that occur as complications and sequelae of pneumococcal pneumonia, or, it may be noted, as independent and primary affections, are inflammations of the pleura, pericardium, and meninges. The last continues to be a significant problem, for the case fatality rate is extremely high, and it does

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not respond to chemotherapy as well as many of the other pneumococcal infections.^{2, 42, 47} Meningitis and otitis media are frequently secondary to pneumonia, and the connection between inflammation of the middle ear and meningeal infection has often been observed.³⁶

There appear to be few, if any, organs or tissues that are not under some circumstances subject to attack. Sinusitis, parotitis, conjunctivitis, peritonitis, and a great variety of other affections are occasionally due to pneumococci. In general, pneumococcal infections of this kind tend to a more favorable outcome than similar infections with streptococci or staphylococci.

Resistance to pneumococcal infection is, in large part, a matter of individual predisposition, and the mere presence of pneumococci in the upper respiratory tract is not sufficient to bring about pulmonary infection. Specific immunity plays a negligible part in resistance, but the complex of factors associated with a state of physiological wellbeing is of the greatest significance. A preliminary depression of resistance by other infections, severe or sudden exposure to cold, fatigue, and other predisposing factors is an almost invariable preliminary to pneumococcal infection. The part played by pneumococcal pneumonia in the fatal termination of many diseases, for example, is well known.

The pathogenicity of pneumococcus types. The case fatality of pneumococcal pneu-

monia is relatively high and indicative of the pathogenicity of these bacteria once they have become established in the lungs. The pneumococcus types differ from one another in this respect; the case fatality in type 1 infections is 25 to 30 per cent, that of type 2 about 40 per cent, that of type 3, 40 to 60 per cent, and that of Group IV infections perhaps 15 to 20 per cent. Because of the relatively recent differentiation of the types comprising Group IV, data on casefatality rates for these types are very meager as yet. The frequency of occurrence of the pneumococcal types in lobar pneumonia and bronchopneumonia is given in figure 89. Here the 10 leading types are: 1, 2, 3, 4, 5, 6, 7, 8, 14, and 19. In some series, type 2 is more frequent than type 3, and in others, including this one, the reverse is true. In a subsequent study of type incidence in the period 1952-1957, the commonest types were 3, 7, and 1, in that order, and in that series type 2 was rare.4

Pneumococcus carriers. As a strict parasite, the pneumococcus is found in man rather than in his environment. Healthy carriers of these bacteria are common; 40 to 60 per cent of groups of persons examined have been found to harbor pneumococci in the upper respiratory tract. This proportion is variable, being relatively high during the cold months of the year and higher among groups of contacts than among noncontacts. The carrier state is not permanent but rather sporadic and intermittent; many

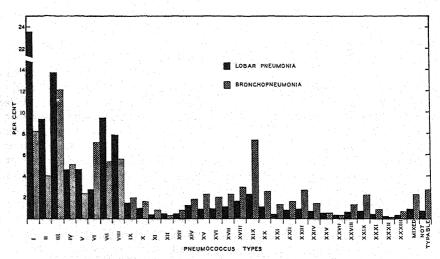


Figure 89. The incidence of pneumococcus types in lobar and bronchopneumonia, observed in a two-year survey in California, Colorado, Illinois, Louisiana, New Jersey, and Missouri, in which pneumococci from 12,447 cases of lobar pneumonia and 3847 of bronchopneumonia were typed. The relative predominance of type 3 over type 2 is sometimes observed. Note the more common occurrence of higher types in bronchopneumonia, the relative incidence of mixed types; *i.e.*, those reacting to more than one antiserum, and the incidence of types other than the first 33. (Data from Rumreich *et al.*)

Pneumococcus Carriers*

PERSONS EXAMINED						INCIDENCE OF CARRIERS							
			Cases	Found		PE	RCENT	of In	ICIDEN	CE OF TYP	ES		
		TOTAL		PER CENT		TOTAL	. 1	2	3	GROUP IV			
Se Se	Non-contacts Contacts	2332 1782	1000 977	42.9 54.8		1027 1018	0.5 3.3	0.9 2.7	8.4 10.0	34.2 41.0			

^{*}Modified from Heffron.21

persons may carry these bacteria for a short time, particularly while having colds and other infections of the upper respiratory tract, while others may carry them for longer periods. There is also a seasonal fluctuation in that increased numbers are found in the winter months.

The great majority of pneumococci found in carriers are the relatively less virulent types of Group IV. Types 1, 2, and 3 are much less frequently found, but an appreciable portion of the population may harbor these types at any one time (see table). In the study made by Smillie, Calderone, and Onslow,⁴⁴ almost every type of pneumococcus was encountered; many individuals carried two or more types simultaneously. The types most commonly found were 3, 7, 21, 25, and 11 in that order of frequency. It is not clear whether an appreciably effective immunity is developed by the carrier.

The epidemiology of pneumococcal pneumonia. Pneumococci are disseminated chiefly through the secretions and discharges of the mouth and upper respiratory tract by direct contact between persons. Droplet infection undoubtedly plays a large part in the transmission of these microorganisms and perhaps accounts for the seasonal incidence of the disease and the increased frequency of carriers during the cold months of the year.

While it is obvious that pneumococcal infection is always exogenous in the last analysis, for practical purposes it is probably endogenous in a large proportion of cases. With a high carrier rate pneumococci are frequently present in the normal individual, and when resistance is reduced to a sufficiently low level they are able to set up an infection. The high incidence of pneumo-

cocci of types 1 and 2 as contrasted with the low carrier incidence of these types, together with the occurrence of seeming epidemics of pneumococcal pneumonia on a small scale, has been regarded by some as evidence of exogenous infection. It is more probable, however, that the greater virulence of some pneumococcus types operates as a selective factor to disturb the random distribution of types in pneumococcal pneumonia. It is known, for example, that minor respiratory infections may be common or epidemic in small groups such as a family. If a given type of pneumococcus of high virulence invades and spreads within the group so that a high proportion of the individuals become carriers, the operation of factors which reduce resistance in the group may result in one or more members coming down with pneumonia due to the type carried. Such a sequence of events was observed in a group of children in a nursery.46 The group was invaded by a virulent type 14 pneumococcus which caused no harm, but when the individual developed an acute respiratory infection, the dormant pneumococcus spread to the middle ear, conjunctivas, and lungs. Such children were sent to the hospital ward and carried the pneumococcus, which spread to most of the children there. Again, the infection was activated, sometimes with serious consequences, on the development of respiratory infection. Under appropriate circumstances pneumococcal pneumonia may occur in epidemic form. One such epidemic, occurring among military personnel, which was studied in detail,24 showed too that the most significant factors were the carrier rate, and the rate of nonbacterial respiratory disease as a predisposing factor.

Other epidemiological characteristics of

[†]The incidence of types is greater than the incidence of carriers because in some instances more than one type was found.

pneumococcal pneumonia include seasonal incidence, which corresponds roughly with the carrier rate; the age incidence, characterized by high morbidity and mortality in infants and the aged; the higher incidence in males than in females; and the apparent greater susceptibility of the Negro as contrasted with the white race.

Bacteriological diagnosis of pneumococcal infections.32 The pneumococcus may be isolated by culture or animal inoculation from specimens such as sputum, pleural exudate, blood, spinal fluid, and pus. Blood agar is the medium of choice, the bacteria growing up in 24 hours as small colonies surrounded by a zone of green hemolysis. It is not possible to distinguish them from α -hemolytic streptococci by colonial or microscopic morphology, but differentiation may be made by the fermentation of inulin and bile solubility of the pneumococcus and its immunological reactions. Blood specimens are cultured in buffered dextrose veal infusion broth, containing 5 mg. per 100 ml. of p-aminobenzoic acid if the individual is undergoing sulfonamide therapy. A portion of sputum, washed in three changes of sterile saline and emulsified in saline, may be inoculated intraperitoneally in a white mouse. With virulent strains the animal will show signs of illness in five to eight hours, and microscopic examination of smears of peritoneal exudate will show large numbers of encapsulated diplococci.

The pneumococcus is identified and typed with antiserum, usually by the Ouellung reaction though agglutination and precipitin tests may be used also. When large numbers of the bacteria are present in sputum, the typing may be done directly without culture or mouse inoculation, but since the Quellung reaction is inhibited in the presence of large amounts of SSS, negative reactions are not significant. Typing is readily carried out, as indicated earlier, on pneumococci present in mouse peritoneal exudate cultures but is no longer carried out except for special purposes, since type-specific serum therapy has been practically entirely supplanted by chemotherapy.

Chemotherapy. The pneumococci are uniformly susceptible to the sulfonamides and the commonly used antibiotics. The incidence of naturally occurring drug-resistant strains has been negligible, and determination of drug sensitivity is not yet an essential part of the chemotherapy of these

infections. *In vitro* the pneumococci are approximately as sensitive to these drugs as is *Str. pyogenes*. In pneumococcal meningitis, combined therapy with sulfonamide and penicillin has been reported to give better results than either alone; it has been reported that sulfonamide reduced the case fatality rate to 73 per cent, penicillin to 50 per cent and combined therapy to 29 per cent.

Pathogenicity for lower animals. The susceptibility of the usual laboratory animals to pneumococcal infection is variable. ranging from the highly susceptible mouse and rabbit through the less sensitive guinea pig to the cat, dog, chicken, and pigeon, which are highly resistant. Rare instances of naturally occurring infections in the usual experimental animals have been reported; for example, an epizootic of type 19 pneumococcal infection in the guinea pig has been described,26 and one of type 2 infection in laboratory rats.34 Animal experiments with the pneumococcus present an example of the general law that susceptibility is characterized by general septicemic infection. resistance by the occurrence of a localized process. The mouse and the rabbit³⁸ develop rapidly fatal septicemia, and in these animals lung lesions, when they occur at all, are slight and usually limited to the bronchopneumonic type. It is possible to produce typical lobar pneumonia in the rabbit by carefully balancing the susceptibility of the animal and the virulence of the bacterium through the use of attenuated cultures or previous partial immunization.

Resistant animals, such as the dog, show an approximation toward the type of pneumococcal infection observed in man, and lobar pneumonia may be produced in monkeys by intratracheal inoculation. The lesions produced in monkeys were considered identical with those in human lobar pneumonia. It is of some interest that pneumococci were found in the blood within six hours after their introduction into the trachea and before the signs of pneumonia appeared, suggesting the bronchogenic rather than the hematogenous origin of the infection.

Man, therefore, may be regarded as an animal of rather high normal resistance. This resistance may, however, be so reduced as to permit the production of localized manifestations, which in still more susceptible individuals may lead to fatal septicemia. In some cases death is due to

overwhelming interference with respiration caused by the local pulmonary lesions, and in others to a toxemia.

Immunity. Experimental animals may be actively immunized against pneumococcal infection by the injection of vaccines of the smooth, virulent bacteria, although the immunity is not of long duration, i.e., not more than a few months. The development of the immune state is accompanied by the appearance of antibodies, precipitins, agglutinins, and the like, as well as a protective quality in the blood serum. In man the situation is somewhat obscure; there is undoubtedly an intimate relation between recovery and the appearance of humoral antibodies, but immunity following infection is slight and transitory, and one attack may succeed another after a short interval. Active immunity in experimental animals is typespecific, however, and it is not improbable that under natural conditions different immunological types may participate in successive attacks.

The question of active immunization of man to pneumococcal infection is of continued interest. Results suggestive of its value were obtained from mass inoculation studies in 1918-1919 in this country. It has been shown²² that the inoculation of typespecific polysaccharides induces an antibody response which reaches a peak within six weeks and persists for about six months: booster inoculation during the following 18 months was not effective in raising the diminishing titers. It has been found³³ also that such immunization significantly reduced both carrier rate and cases of infection with the pneumococcus types immunized against, though not the incidence of other types. In the aggregate, then, these data suggest that an appreciable degree of effective immunity may be produced by immunization procedures.

The results of the therapeutic use of antiserum are variable, being excellent with some types of pneumococci and not with others. Serum therapy was formerly of great practical importance, but with the introduction of effective chemotherapeutic agents is no longer used.

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 243
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Chapter Nineteen

THE GRAM-NEGATIVE PATHOGENIC COCCI (NEISSERIA)

The Gonococcus and the Meningococcus

The gonococcus and the meningococcus are the chief representatives of a small group of closely related bacteria whose other members are nonpathogenic inhabitants of the mouth and upper respiratory tract of man.⁵⁸ Two groups of species are separated from

one another on the basis of pigment production, and further differentiation is made by means of fermentation reactions. Pigmented varieties are frequently found in the nasopharynx (Neisseria flava I, II, III).

The Gonococcus

Neisser in 1879 first called attention to the constant presence of a peculiar coccus in gonorrheal pus. In cases of gonorrhea of recent origin this was the sole organism found; it not only occurred in the urethral and vaginal discharges of ordinary gonorrhea, but was present in the exudate in conjunctivitis due to gonorrheal infection. Pure cultures of this microorganism were isolated in 1885 by Bumm, who succeeded in demonstrating its etiological relation to gonorrhea by the inoculation of human volunteers. This bacterium, known generally as the gonococcus, has been termed Micrococcus gonorrhoeae and Diplococcus gonorrhoeae, but the genus Neisseria is now more or less generally accepted, and this bacterium is properly known as N. gonorrhoeae.

Morphology and staining. In preparations made from gonorrheal pus the cells of the gonococcus occur in pairs, with the flattened sides in juxtaposition; the appearance in stained preparations resembles that of a coffee bean. In pure culture the cocci appear

as oval or spherical and are often aggregated in irregular masses without the typical diplococcus arrangement. In pus smears the gonococcus occurs almost entirely within the leucocytes; frequently enormous numbers may be found packed within a single phagocyte. In the earliest stages of infection gonococci may be found extracellularly, and the same is true of cases of gonorrhea of long standing. The gonococcus is nonmotile and does not form spores.

The colonies of the gonococcus are small, translucent, finely granular with lobate margin and grayish white with a pearly opalescence when viewed by transmitted light. Larger colonies may be formed on special mediums. Colonial appearance is, however, subject to variation (see below).

Unlike the other pyogenic cocci, the gonococcus and related forms are gram-negative, a staining characteristic that is of considerable diagnostic value, since it serves to differentiate the gonococcus from other cocci present in the urethral or vulvovaginal

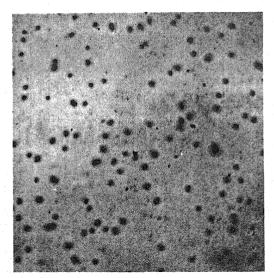


Figure 90. The gonococcus from pure culture. Fuchsin; \times 1050.

tracts. Other gram-negative cocci may be found occasionally, sometimes within the leucocytes, but they are rare. The tendency to decolorization in the Gram stain is variable. Some strains decolorize much more readily than others, and gonococci embedded in masses of pus may retain the stain; hence the preparation of thin, uniform films is highly desirable. The gonococcus stains with the aniline dyes, but polychrome stains, such as Pappenheim's stain,² are more useful. Intracellular granules may be found in stained preparations, but in general the gonococci from young cultures stain evenly, while older cultures (24 hours and older) contain large swollen involution forms, which may stain poorly.

Physiology. In its nutritive requirements the gonococcus is one of the most fastidious bacteria, particularly upon primary isolation, and an enriched medium is required for cultivation. Earlier mediums were enriched by the addition of ascitic and hydrocele fluid. Chocolate (heated blood) agar has been the most widely used, and the basal medium may be enriched with horse plasma and hemoglobin, and sometimes Nile blue A is added.

The nutritional requirements of the gonococcus are complex although some strains may be grown on a tryptic digest—glucose agar containing added cystine, and relatively complex synthetic mediums have been devised which will support growth.²⁸ Stock strains are commonly less exacting than recently isolated strains, and it is likely that growth requirements are more accurately reflected in the mediums required for isolation or growth of fresh isolates. Such strains do appear to require added glutamine and cocarboxylase since they apparently cannot phosphorylate thiamin, and glutathione is required by some strains.

A sufficient supply of moisture is essential; there should be water of condensation on the tubes or plates, and the atmosphere of the incubator should be kept saturated with water. Incubation in an atmosphere of increased CO₂ tension, about 10 per cent, greatly improves growth and is a practical necessity in primary isolation.²²

With continued cultivation on laboratory mediums, the gonococcus appears to become somewhat less fastidious, and some strains may eventually grow upon the ordinary infusion mediums. The preservation of cultures is difficult, however, for the gonococci die off in two to three days at room temperature and in six to eight days at 37° C., but will live longer when kept in the cold. Even upon continued transfer the gonococci die off, and cultures are often lost. The optimum temperature for growth is 37° C., growth does not occur below 30° C., and temperatures of 40° to 41° C. are definitely harmful. The gonococcus will grow under anaerobic conditions but is essentially aerobic in character

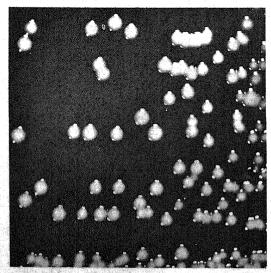


Figure 91. Colonies of the gonococcus on blood agar, × 6.

Fermentation	Reactions	of the	Gram-negative	Diplococci
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NONPIGMENTED SPECIES	DEXTROSE	MALTOSE	SUCROSE	LEVULOSE	MANNITOL
N. gonorrhoeae (gonococcus)	+	_			
N. intracellularis (meningococcus)	+	+	-	-	
N. catarrhalis		_	_	Marine.	
N. sicca	+	+ ' - '	+	+	· · · · · ·
PIGMENTED SPECIES					
N. perflava (flava I)	+	+	+	+	+
N. flava (flava II)	+ '-	+	- · · · · · · · · · · · · · · · · · · ·	+	
N. subflava (flava III)	14, 1	+ ,		1985 - - 1985	-
N. flavescens	-				-

With respect to deleterious influences the gonococcus is a delicate microorganism. It is readily killed by heat, as indicated above, and by dilute antiseptics; 1 per cent phenol, for example, kills in one to three minutes. It is remarkably sensitive to certain of the flavine dyes and is rapidly destroyed by silver salts.

The gonococcus is sensitive to drying and, under ordinary conditions, can survive exposure to the air for only a very short time—one to two hours—although in masses of dried pus it may live exceptionally for six to seven weeks. Unlike most gram-negative bacteria, it is sensitive to penicillin as well as to streptomycin and the tetracyclines.

The gonococcus is not very active biochemically. Glucose is fermented, principally to lactic acid, but many other sugars

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are not attacked, indol is not produced, nitrates are not reduced, and no change is produced in litmus milk. Its fermentation of glucose and failure to ferment maltose distinguish it from the other nonpigmented Neisseria; the fermentation reactions are reliable and used for purposes of identification.48 but the basal medium used must be such as to support growth of the microorganism.31 Catalase is produced, and a characteristic that has been turned to practical differential use is the formation of indophenol oxidase. The specimen is cultured on 10 per cent heated blood (chocolate) agar in an atmosphere containing 8 per cent carbon dioxide, followed by 24 hours' incubation in air. A 1 per cent solution of tetramethyl-p-phenylenediamine is poured on the incubated plate and poured off again

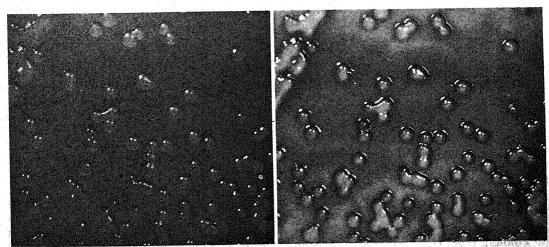


Figure 92. The oxidase test for the identification of gonococcus colonies. Pure culture on blood agar. Left, gonococcus colonies before the application of tetramethyl-p-phenylenediamine solution. Right, the same colonies after the application of the reagent. Note the greater intensity of color about the edges of the colonies immediately after application and the discoloration of the medium. × 5.

immediately or sprayed on with a nasal atomizer. Colonies of bacteria forming indophenol oxidase turn a bright purple color. The bacteria are not immediately killed and may be subcultured within half an hour. This so-called oxidase reaction, coupled with the examination of smears for gramnegative intracellular diplococci, is generally used in the laboratory diagnosis of gonorrhea.

Toxins. Other than a weak hemolysin, the gonococci apparently form no extracellular toxic substances, but the cell substance is toxic to experimental animals on parenteral inoculation and has been reported to produce suppuration when instilled into the human urethra. The toxicity is extractable in dilute alkali as a "nucleoprotein," and from this material or from the intact bacteria by trichloracetic acid or diethylene glycol, in the latter instance behaving like a Boivin antigen, e.g., the endotoxin of the gram-negative bacilli. It was subsequently found to be extracted in phenol by the Westphal method to give a toxic lipopolysaccharide which could then be purified by fractional precipitation with acetone. This substance is similar to the gram-negative bacillus endotoxins as typified by colon bacillus endotoxin, but on analysis has been found to differ significantly in lacking diaminopimelic acid and glycine and in lesser content of other amino compounds.65 The contained carbohydrate has been found to consist of D-glucosamine, glucose, and galactose, the amino acids presumably serving to link the sugar and lipid components in the intact molecule. The pharmacological activity of the gonococcal endotoxin appears to be nonspecific and overtly similar to that of the other endotoxins. Its role in the pathogenesis of gonococcal infections is uncertain.

Variation. Numerous workers have observed that the cultural characteristics of the gonococcus are subject to considerable variation. It has been found that two types of colonies may be observed which appear to be correlated with immunological type. The one, designated as type I, is a large, irregular, flattened, translucent colony which appears dark gold in color by oblique transmitted light.³³ On continued incubation papillae appear on the surface. The other type of colony, type II, is somewhat smaller, round, raised with slightly convex uneven surface, and opaque. Type I colonies are generally recovered from acute gonorrhea, while type II are found in old laboratory strains and, sometimes, in chronic gonorrhea. The papillae are thought to represent the first step in transition toward the type II form. The relation of these forms to the dissociative changes of other bacteria is uncertain. Small colony variants, presumably arising from a dissociative process, have been produced experimentally and have also been found on primary isolation from clinical material.⁴⁷

Drug resistance. Resistance of gonococci to the sulfonamides and antibiotics is readily acquired under experimental conditions, and resistant strains appear in nature coincident with the general application of chemotherapeutic agents. When the sulfonamides were introduced into general use as chemotherapeutic agents in 1936-37, 80 to 90 per cent of infections could be cured with them. An increase in the proportion of refractory infections became noticeable in three or four years, a sharp increase occurred in 1942-43 from 25-35 per cent to 50 per cent, and by the late 1940's as few as 15 per cent of infections in some areas responded to sulfonamide therapy.74 Gonococcus strains isolated from refractory infections usually prove to be resistant on laboratory examination, many, but not all, of them producing p-aminobenzoic acid in excess amounts.

Meantime penicillin therapy became available in the middle 1940's and has been widely used, usually as repository penicillin, except in France where streptomycin has been preferred to avoid masking of concurrent syphilitic infection. Subsequently other antibiotics, the tetracyclines, erythromycin, chloramphenicol, etc., effective in the treatment of gonorrhea, became available also but have not been generally used. Strains of gonococci resistant to penicillin did not appear in increased numbers until about 10 years after its introduction, but by the late 1950's it was apparent that 20 per cent or more of strains isolated in many areas were resistant to high concentrations, 20-fold or more the original sensitivity, not ordinarily achieved as blood levels. 6, 8, 41, 56, 68 A temporary, and effective, expedient has been the use of larger doses of the antibiotic. Concurrently, strains of gonococci resistant to streptomycin have increased in number in France, with 75 per cent of strains resistant to 50 μ g./ml. or more, and more than 20 per cent resistant to 1000 µg./ml., in contrast to an original sensitivity level of perhaps 5-10 μg./ml. 16, 57

Increase in the proportion of resistant strains coincides with the use of the chemotherapeutic agent. There seems to have been no significant increase in penicillin-resistant strains of gonococci in France, nor an increase in streptomycin-resistant strains in other European countries and the United States where penicillin has been used. The tendency to reversion to sensitivity when the chemotherapeutic agent is discontinued. apparent in some other bacteria, e.g., the staphylococci, is indicated by the decline in the proportion of sulfonamide-resistant strains, which by 1958 reached approximately the level which prevailed when sulfonamide therapy was generally introduced.10

Clinical refractoriness to penicillin therapy is not necessarily always a consequence of infection with a resistant strain of gonococcus. Some cases may be refractory because of failure of the antibiotic to affect phagocytosed gonococci, which have been shown experimentally to survive considerable increases in concentration of penicillin over the minimal inhibitory concentrations. Further, the picture of relative efficacy of chemotherapy may be clouded by the by-no-means uncommon occurrence of nongonorrheal urethritis, 27, 50, 51 which may not be affected by the chemotherapeutic agents used.

Antigenic structure. The gonococci are apparently of uncertain antigenic structure as assayed by conventional methods of antigenic analysis.54 Certain of the antigens appear to be thermostable, and thermostable and thermolabile partial antigens occur also, some of which may function as blocking antigens, and antigenicities appear to be shared with meningococcus and some strains of Pasteurella. Gonococcus strains not only differ when freshly isolated, but also apparently change antigenically on continued subculture. Studies with soluble antigen, prepared by extraction with dilute alkali and precipitation with ethanol from neutral solution, in the passive hemolysis reaction have given more consistent results. Two main antigens are demonstrable by this technique, the one associated with colonial type I which is shared with meningococcus, and the other with type II. The dual nature of the antigenicity is also indicated by differentiation of the antigenicity which sensitizes red cells to immune lysis in the presence of antiserum from that which fixes comple-

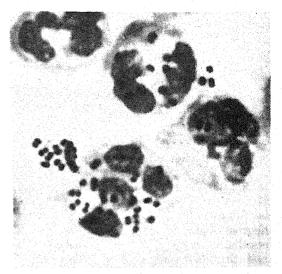


Figure 93. Urethral smear from gonorrhea. Note the intracellular and extracellular positions of the gonococci and their typical coffee-bean shape and arrangement in pairs. Gram stain; × 2400.

ment. The relation of this antigenicity to the lipopolysaccharide endotoxin has not yet been described, but it is heat-stable and presumably polysaccharide in nature. Antiserum to the alkali-extracted endotoxin described above is protective in the mouse.

Further study has shown that the heatstable antigenic complex contains at least six antigens shared with the meningococcus, and which occur in varied combinations,78 and also that freshly isolated strains of gonococcus are often inagglutinable in antiserums to these antigens.⁷⁷ The last has been taken to suggest that fresh isolates may contain a K-type antigen (Chap. Twenty), labile in that it is readily lost, and functioning as a blocking antigen. The existence of this kind of antigen in the gonococcus has been demonstrated by the fluorescent antibody technique.¹³ It appears to be sharply specific for the gonococcus, and is present in a relatively large amount on gonococci in acute infections, i.e., as observed in urethral smears, and in very young cultures, but has largely disappeared in 30-hour cultures.

Pathogenicity for man.²⁶ Few diseases are so widely disseminated through all classes of society as gonorrhea. Precise information as to the incidence of the disease is not available. It is estimated that in the United States no more than 10 to 20 per cent of cases are reported, and that gonorrhea is the third most common infection (after mea-

sles and streptococcal infections).²⁵ In 1944 it was predicted that, with the means, penicillin, available, the virtual eradication of gonorrhea could be anticipated. In that year 301,000 cases were reported, and the number rose to 401,000 in 1947, subsequently declining somewhat to stabilize, since 1952, at about 220,000 cases reported per year. More than half of these are persons under 25 years, with a modal age of 18 for females and 22 for males.⁶

The gonococcus usually gains entrance to the tissues after deposition on the surface by burrowing through the superficial epithelium, between the cells, to reach subepithelial connective tissue from which it spreads by direct continuity or by the lymphatic or blood vessels. Stratified squamous epithelium is more resistant to this penetration than columnar epithelium. The characteristic reaction within the tissues is a dense cellular infiltration of polymorphonuclear leucocytes, plasma and mast cells, and these are eventually replaced by fibrous tissue. In the male, epididymitis, chronic urethritis and stricture, and other inflammatory conditions develop, and in the female, the entire genitourinary tract may be involved, and the fallopian tubes, the ovaries, and the peritoneum are not uncommonly invaded. The gonococcus may also invade the blood stream from local lesions and be carried to various parts of the body to give rise to a variety of extragenital

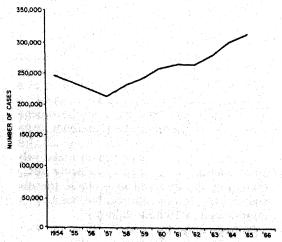


Figure 94. The prevalence of gonorrhea in the United States as indicated by reported civilian cases during the period 1955-1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

lesions. Especial predilection is shown for the synovial membranes of the joints, where it causes the so-called gonorrheal rheumatism, and for the heart valves, where it produces endocarditis. Local or general complications occur in perhaps 30 per cent of all cases. Gonococcal meningitis occurs, perhaps more frequently than formerly thought. There has been a general belief that the incidence of complications—epididymitis, prostatitis, salpingitis and arthritis—has been less since the introduction of effective chemotherapy, but this appears to be open to question.²⁰

Once established, untreated gonococcal infection persists for a long time; five to 15 years' duration has been reported. Exclusion of reinfection is a difficult matter, but infection of seven years' duration without reinfection has been observed. Subclinical infection may occur, with or without transient symptoms, and individuals so infected may act as carriers. The clinical disease is often not as overt in the female, and infected promiscuous females constitute an important reservoir of infection.⁷⁶

Epidemiology.³⁵ Gonorrheal vulvovaginitis occurs in epidemic form in little girls, and in these instances the infection is transmitted by bedclothes, towels, common bathtubs, and other fomites. The commonest complications are urethritis, proctitis, and cervicitis. Such epidemics are frequently exceedingly difficult to control and constitute a serious problem in many institutions such as children's wards in hospitals.^{9, 64}

Gonorrheal ophthalmia of the newborn is a well-known consequence of maternal infection; infection does not occur in utero but during passage through the birth canal. Although exact information is not obtainable, it is estimated that 10 per cent of all cases of blindness are traceable to this source and that in the United States there are perhaps 12,000 children blind from this cause. Infection may be prevented by the prophylactic instillation of silver salts such as silver nitrate. Some of the antibiotics, ³⁹ especially penicillin, are equally effective and may be given by the intramuscular route. ¹²

Except in the case of vulvovaginitis in children, gonorrhea is spread by direct contact, usually sexual. Once infected, an individual may remain infective for a long time, and gonococci may persist in the genitourinary secretions for years after apparently

complete recovery, and even though they may not be found by bacteriological examination the infection may be transmitted. Gonorrhea persists, then, in the human population in a smoldering endemic form and, as consequence of the nature of its transmission, widespread epidemics in the usual sense do not occur.⁷⁵

Bacteriological diagnosis of gonococcal infection.^{55, 73} A diagnosis of gonorrhea is established with certainty only by isolation and identification of the causative microorganism. The latter assumes significance in view of observations such as those of Johnston,³⁰ who found that 35 of 43 cultures were Neisseria other than the gonococcus, and the incidence of 1.5 to 3.4 per cent of Neisseria other than gonococcus reported by Wilkinson.⁷² In fact, nongonococcal urethritis may be more prevalent under certain conditions than gonococcal infection, the condition being associated with Mycoplasma.

While it has been said to be "primitive" by some workers, the demonstration of intracellular gram-negative diplococci in direct smears constitutes a presumptive, but not substantiated, diagnosis of gonorrhea. The validity of such diagnosis is enhanced by the application of fluorescent antibody staining to such smears.^{34, 46, 71} Diagnosis on the basis of stained smears is less reliable in the female than in the male, as is culture also.

It is generally agreed that culture of the gonococcus gives a higher proportion of positive results than direct smear examination alone, and cultured gonococci may be identified. The question of survival of this relatively delicate bacterium in transport of specimens is of some importance. Stuart's transport medium⁶³ is considered to be by far the most satisfactory. For culture an infusion chocolate agar, developed by McLeod, or minor modifications of it such as the plasma hemoglobin modification studied by Thayer, Schubert, and Bucca⁶⁶ is a medium of choice, and is inoculated directly with the specimen (or sediment if it is urine or spinal fluid). The culture must be incubated in 10 per cent CO₂; this atmosphere may be satisfactorily approximated by putting the plates in a jar together with a lighted candle and sealing or by the inclusion of a handful of moistened fresh oats in a sealed container with the cultures. Or carbon dioxide may be evolved from H₂SO₄ and an excess of bicarbonate, the amount of the former being determined by the volume of the container to a v/v concentration of 8 to 10 per cent. The oxidase test, carried out preferably by spraying the plates with the reagent from an atomizer or by pouring it on and off quickly, serves to differentiate the oxidase-positive colonies and, if picked immediately, they may be subcultured. Identification is based upon sugar fermentations in serum broth or serum agar containing an indicator. The fluorescent antibody staining technique may be applied to smears of pure cultures so isolated, as well as to direct smears as indicated above. 19

Chemotherapy. As described above, the occurrence of resistance to sulfonamides and antibiotics markedly affects successful chemotherapy. Disregarding such resistance, the gonococci are sensitive to sulfonamides, usually sulfadiazine, and to the antibiotics penicillin, erythromycin, tetracyclines, chloramphenicol, carbomycin, streptomycin, neomycin, and bactracin in that order, on the basis of activity per unit weight. Since streptomycin, which occurs far down on the list, is effective often in a single dose, there is a variety of available chemotherapeutic agents.

Pathogenicity for lower animals. The gonococcus is nonpathogenic for lower animals, aside from the toxicity of the cell substance as noted above, and gonorrhea has never been reproduced in experimental animals, including anthropoid apes. An experimental infection of the anterior chamber of the rabbit's eye has been described by Miller and his co-workers^{15, 44} in which the gonococci multiply and invade intraocular tissues, especially the ciliary body and lens, giving rise to a chronic infection in approximately one-third of the animals. This infection has been made use of in the study of the efficacy of chemotherapeutic agents.

Immunity. Little if any immunity to the gonococcus is acquired as a result of infection, and second and third infections may be superimposed upon the first, i.e., acute upon old chronic infections. As might be expected, then, the therapeutic use of vaccines and various types of antiserums is without effect. The significance of the observed extensive phagocytosis of gonococci by polymorphonuclear leucocytes is uncertain

Some degree of immunological response is evident, however. Complement-fixing antibodies are usually present, and patients may give a marked skin reaction to suspensions of killed gonococci. A number of attempts have been made to utilize these responses in the immunological diagnosis of gonorrhea. The complement-fixation test has shown some promise but is not generally used. The skin reaction is apparently too

variable to have practical value. It has also been observed that the discharges from gonorrheal inflammation give a precipitin reaction with antigonococcus serum, but this flocculation reaction has as yet no diagnostic value.

The Meningococcus

Inflammation of the meninges or investing membranes (pia-arachnoid) of the brain and spinal cord may be provoked by a variety of microorganisms and may occur either as a primary affection or secondarily in the train of an infection originally begun elsewhere. One form of meningitis, characterized especially by epidemic spread and usually designated as epidemic cerebrospinal meningitis, spotted fever, or cerebrospinal fever, is caused by a specific microorganism commonly known as the meningococcus.

This bacterium was described by Marchiafava and Celli in the meningeal exudate as early as 1884, but the first important work upon it was that of Weichselbaum, who, in 1887, obtained it in pure culture and described it in detail as the characteristic micrococcus found in six cases of acute cerebrospinal meningitis. Confirmation was supplied by the work of Jäger in spite of some faulty observation.

The meningococcus has been designated by a variety of names, including *Micrococ*-

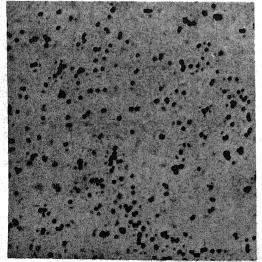


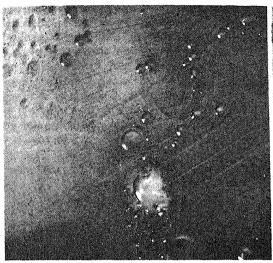
Figure 95. Meningococcus, pure culture. Note the typical diplococcus arrangement. Fuchsin; × 1050.

cus meningitidis, M. intracellularis meningitidis, Neisseria intracellularis, and, according to Bergey, N. meningitidis. Although Neisseria is generally accepted, common usage is divded between N. intracellularis and N. meningitidis.

Morphology and staining. In film preparations of the meningeal exudate the meningococcus is very like the gonococcus and occurs in pairs or tetrads both within the leucocytes and free. The diplococci are flattened toward one another like gonococci. and there is considerable variability in the size of different cells in the same smear. In cultures the meningococcus averages a little less than 1 μ in diameter and appears, as a rule, in pairs; short chains are seen more rarely. The variability in size observed in meningeal exudate may also be seen in cultures, particularly in those more than 24 hours old. Involution forms are common. and it is not unlikely that the larger cells are degenerative. Capsules are usually not apparent but become swollen in the presence of specific immune serum—the Quellung reaction. The meningococcus is nonmotile and does not form spores.

Meningococcus colonies in blood agar are moist, elevated, and smooth, and have a bluish gray tinge. They do not produce green discoloration or hemolysis and may be readily differentiated from the hemolytic and viridans streptococci and the pneumococcus. The colonies are not so white and opaque as those of the staphylococci.

The meningococcus stains readily with the usual aniline dyes and, like the gonococcus, is gram-negative. The involution forms found in cultures tend to stain unevenly, but even young cells may show the presence of metachromatic granules when stained by Löffler's alkaline methylene blue and other stains, and to a greater extent than the gonococcus. No sure distinction between the meningococcus and the gonococcus can be made on morphological grounds, and the identification of gonococci in gonococcal meningitis



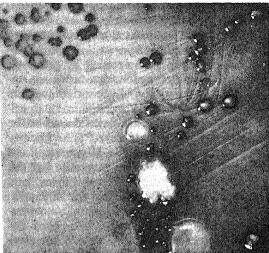


Figure 96. The oxidase test for the identification of meningococcus colonies. Mixed culture on blood agar. Left, colonies of meningococci and contaminants before the application of tetramethyl-p-phenylenediamine solution. Right, the same colonies after the application of the reagent. Note that the meningococcus colonies show the development of color first about the edges and there is slight discoloration of the medium. × 5.

is dependent upon culture and differential fermentations.

Physiology. Strains of meningococci vary considerably in the ease with which they may be cultivated; some strains will grow, although sparsely, on nutrient and infusion mediums but, in general, rich mediums containing serum or whole blood are required. Infusion base chocolate agar and blood agar are the most useful mediums. The nutritive requirements of meningococcus are similar to those of gonococcus, but some strains do not seem to require added cystine. Stock strains have been grown on synthetic mediums containing glutamic acid and cystine, or glutamic acid and lactate or glucose, and occasional freshly isolated strains will grow on these mediums.24,59

In isolating the meningococcus it is essential that the culture medium be warm when inoculated and kept warm until finally placed in the incubator. Growth is favored, especially in primary isolation, by incubation in an atmosphere containing 10 per cent carbon dioxide. The meningococcus will grow over a temperature range of 25° to 42° C., with an optimum at 37° C. Although sparse growth will occur under anaerobic conditions, the meningococcus is, for all practical purposes, an aerobe.

Continued cultivation on laboratory mediums results in more luxuriant growth, and the bacteria presumably become less nutritionally fastidious. Meningococcus cultures are difficult to keep, however, and tend to die out in stock culture. In most mediums these bacteria die within a few days if not transferred, but vitality may be preserved for several weeks in stab cultures in starch agar (1 per cent cornstarch in nutrient agar) and are best kept in the incubator.

The relatively early appearance of involution forms in meningococcus cultures as well as their limited viability when not transplanted is, perhaps, attributable to their formation of an active autolysin, and in saline suspension in the incubator autolysis may take place within a few hours. The autolysin is heat-labile, being destroyed at 65° C. in 30 minutes, and suspensions prepared for agglutination studies should be inactivated in this way.

The meningococcus, like the gonococcus, is a delicate microorganism and not highly resistant to deleterious influences. It is killed in a short time by drying and by exposure to dilute disinfectants. It is particularly sensitive to heat and cold and, unlike many bacteria, dies out within a few days at 0° C.

The meningococcus is not an active fermenter. Considerable quantities of acid, presumably lactic for the most part, are formed from glucose and maltose. The fermentation of maltose serves to distinguish the meningococcus from the gonococcus.

Neither is this bacterium actively proteolytic, for coagulated serum is not liquefied.

Toxins. Meningococcal meningitis in man and that reproduced in experimental animals are usually accompanied by profound toxemia. The meningococcus, however, appears to form no soluble toxin, though its cell substance is toxic to experimental animals and the toxin has been found to be lipopolysaccharide in nature.⁴² The toxicity is heat-stable (100° C. for 30 minutes) and the rate of its destruction suggests that the endotoxin consists of two substances, one much more thermostable than the other.

Variation. Rough and smooth colony types of the meningococcus have been described. Recently isolated strains are generally smooth while old stock cultures are rough. Mucoid colonies are observed. The change from smooth to rough is associated with a partial loss of immunological type specificity.

Classification. The meningococci are closely related to the gonococci, not only morphologically and physiologically but immunologically in that certain antigenic substances appear to be held in common as indicated previously.⁷⁸

The meningococci are not antigenically homogeneous, and occur as several serotypes, demonstrable by the agglutination and Quellung reactions, 45 which are determined by polysaccharide haptenes except one, the P substance, which is a toxic protein. The polysaccharides of type I (type A)³²

and type C (type $II\alpha$)^{69, 70} have been prepared in purified form, and the latter has been found to contain sialic acid and hexosamine. The relationship of the P substance to the endotoxins is not known. Differentiable types have been described by a number of workers since 1914, using different systems of notation. This has resulted in some confusion, and an internationally acceptable recommendation in which the serotypes are designated types A, B, C, and D has been agreed upon.⁵ The various systems of nomenclature are illustrated in the accompanying table.

While the routine typing of meningococci is probably not worth while, typing in connection with epidemiological studies, *i.e.*, the distribution of types occurring in healthy carriers as well as in cases of the disease, has been valuable.

Pathogenicity for man.³⁷ The resistance of man to meningococcal infection is relatively high, and the incidence of healthy carriers is invariably considerably higher than that of cases of the disease. It is probable that predisposing factors play a large part in determing whether or not infection will occur; insufficient clothing, inadequate ventilation, exposure to inclement weather, and fatigue very likely contribute in large measure to increasing susceptibility.

The meningococcus is initially present in the nasopharynx and from there gains access to the central nervous system. The route by which this occurs is uncertain; it is thought

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The Nomenclature of Meningococcus Types*

DOPTER AND PAURON, 1914	woolstein, 1914	GORDON AND MURRAY, 1915	GRIFFITH AND SCOTT, 1916	PULLON, 1917	NICOLLE, DE BAINS AND JOUAN, 1918	EVANS 1920	COMMON USE SINCE 1940	RECOM- MENDED†
Meningococ-	Normal	I		С				
cus	Irregular	III	1	A	A	R	1	A
Parameningo-	Parameningo-	11	11	В	В	S	11	В
coccus α, β, γ	coccus	IV	11	eğir A	В	Z	IV	D
					С		ΙΙα	С
				1	D#	e e e e par hal	Assault Victoria	

^{*}From Branham.5

[†]By the subcommittee on the taxonomy and nomenclature of Neisseria of the Nomenclature Committee of the International Association of Microbiologists.

[‡]The relation of this type to the others is unknown.

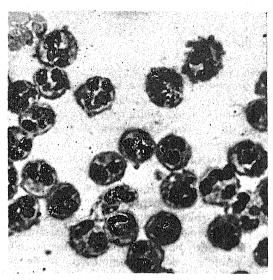


Figure 97. Meningococci in spinal fluid, showing phagocytosis of the microorganisms. Gram stain; \times 1050.

by some that the bacteria follow the perineural spaces of the olfactory nerves or set up a preliminary sinusitis and reach the brain either via the lymphatics or by direct extension through the bone. Others believe the meningococci reach the central nervous system via the blood stream through a preliminary bacteremia. While there is no definitive evidence concerning the means by which meningococci reach the central nervous system from the nasopharynx, the evidence appears to favor the hematogenous route. Occasionally the infection in the nasopharynx may extend into adjacent areas. giving rise to conjunctivitis, pneumonia, or other infections.

In the healthy carrier the infection remains confined to the nasopharynx and in this case is short-lived there and produces few or no symptoms. When the blood stream is invaded early in the disease, hemorrhages usually occur in the skin and petechiae appear, especially on the wrists and ankles, or on any mucous or serous surfaces. These are apparent in 24 hours after invasion and fade in a few days. The rash is quite different from other purpuric rashes: the spots take geometrical shapes and are highly irregular in size. Meningococci may be observed in smears of material taken from these lesions. Other symptoms include sudden onset, chills, fever, and meningeal symptoms such as headache and drowsiness. Pain in the arms and legs is common. The invasion of the blood stream may take the form of a fulminating meningococcemia (the Waterhouse-Friderichsen syndrome) adrenal with apoplexy as the immediate case of death and massive bilateral hemorrhage of the adrenals as the outstanding pathology. 29, 38, 49 This form of meningococcal disease is uncommon -little more than 200 cases have been reported-and occurs much more frequently in infants than in adults. Its sudden and violent character with rapid fatal termination has led to deaths due to this cause being classified as suspicious. The bacteremia may also take a more chronic form, and give rise to a purulent synovitis or meningococcal arthritis.7, 11

Upon reaching the central nervous system. the meningococcus sets up a suppurative lesion of the meninges which involves the surface of the spinal cord together with the base and cortex of the brain. The spinal cord lesions are of two main types during the acute phase of the disease: one is an acute transverse myelitis, and the other a poliomyelitic type. Later there is a diffuse arachnoiditis that constricts the cord and interferes with its blood supply and may result in extensive intramedullary changes. The microorganisms are invariably present in the spinal fluid, which may vary from a slight to a heavy turbidity. The bacteria are found, sometimes in great numbers, both free and within the leucocytes in smears of spinal fluid. The case fatality is variable, but in any case high, ranging from 35 to 80 per cent, and has been reduced to 16 per cent by improved methods of treatment, including chemotherapy.

There is some divergency of opinion regarding the nature and extent of sequelae to meningococcal meningitis, but these appear to include deafness, which is the most common; blindness; and pain and weakness of the neck, arms and legs. In a representative series of cases, for instance, 7 to 8 per cent of the patients showed sequelae persisting for nine to 30 months after recovery.

Epidemiology. 18, 21, 28, 79 Like most of the respiratory infections, meningococcal meningitis is disseminated by direct contact and droplet infection through the secretions of the mouth, nose, and throat. Infection is spread by patients and convalescents to a limited extent, but healthy carriers of meningococci are of primary importance. 52, 53 Some persons are temporary carriers, while

others are chronic, discharging meningococci more or less continously or in a snoradic fashion. Of a group of 10 carriers, half were of the chronic type, carrying what was apparently the same strain of microorganism for over two years. Weekly examination might be negative for a period, in one case as long as four and one-half months, and then the same type of meningococcus would appear. In a continuous survey⁴³ over a 17 month period of hospital staff and emplovees, the peak in carrier rate of 17 per cent was reached in April and May. Of 90 carrier strains isolated, 20 did not fall into the recognized types, and 70 were typable. Of the latter group, 16 were type I, 26 type II. and seven type $II\alpha$. Other studies of carriers have yielded similar results.61 Spouses of carriers were only occasionally carriers and then of a different type of meningococcus, suggesting a limited infectivity of the carrier strains.

The carrier rate is much higher than the incidence of the disease, and the theory that the second is a function of the first has been

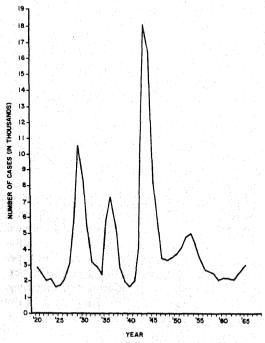


Figure 98. The prevalence of meningococcal infections in the United States during the period 1920–1965 as indicated by the number of cases reported. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

an intriguing one. Studies carried out during World War I suggested that there is a direct connection between the two, and that when the carrier rate exceeds 20 to 30 per cent. cases begin to appear. This relatively precise correspondence has not been substantiated. and the extensive studies of Aycock and Mueller¹ carried on during World War II showed that, while in general the prevalence of meningitis may be attributed to the prevailing type I carrier rate, variations in the incidence of disease between winter and summer, between the military and civilian populations, and between raw recruits and seasoned troops, were not associated with corresponding variations in the carrier rate.

Epidemics of meningococcal meningitis are prone to occur in military populations. and epidemic cerebrospinal fever was, with influenza, one of the most important diseases among the troops in the First World War. The influence of predisposing factors causing an increased susceptibility is especially marked under conditions of military life: fatigue, exposure to inclement weather. and similar factors very likely play a part in reducing the normal resistance of the raw recruit. This general reduced resistance, coupled with the opportunities for the dissemination of the microorganisms provided by the close communal existence in barracks, undoubtedly is an important factor in the genesis of these epidemics. Outbreaks in the civilian population differ in that children (over three months old) and adolescents are generally attacked; susceptibility appears to be greatest in children under 10 years of age, distinctly less in adolescents, and remains at a low level thereafter.

Whether occurring in civilian or military populations, epidemics of meningococcal meningitis have a number of distinctive characteristics. The relatively high carrier rate that may prevail, coupled with the low morbidity, estimated at 0.01 to 0.3 per cent of the exposed population, is an expression of a high degree of normal resistance, while the high case fatality rate indicates the serious course of the infection once established. The relative insusceptibility of the general population also results in the spotty character of the spread of the disease; some groups escape which are closely associated with the focus of infection, while in others, apparently remote, outbreaks occur. Direct transmission from case to case is not commonly observed. Usually carriers constitute

the link between cases. Further, epidemics frequently consist of a series of recurring outbreaks rather than the well-marked single epidemic wave often observed to occur in other diseases. In this country during the past five decades meningococcal meningitis has occurred in epidemic waves at six to 12 year intervals. Since 1925 there have been three major epidemics, centering in the years 1929, 1935, and 1943. Following 1943, there was a steady decrease in the numbers of cases and deaths until 1951. when the occurrence of a slightly greater number of cases of meningococcal infection suggested the possibility that another period of increased incidence might be developing. The incidence did, in fact, rise in the United States to a peak of 5077 cases in 1953 but subsequently declined to 2150 cases in 1962. Subsequently the number of reported cases increased to 2470 in 1963, 2825 in 1964, 3040 in 1965, and, provisionally, 3373 in 1966.

The seasonal incidence is marked in temperate climates. Males are more frequently attacked than females, although this may be an expression of risk rather than sex differences in resistance. Racial differences in susceptibility are indicated by military experience in which the morbidity and mortality rates of the colored troops were twice those of the white.

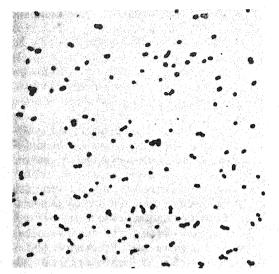
Chemotherapy. the chemothera-Of peutic drugs presently available, the sulfonamides, especially sulfadiazine, remain the drugs of choice. The meningococcus resembles the gonococcus in its in vitro sensitivity to the antibiotics, but these generally diffuse poorly and do not readily enter the central nervous system; penicillin may be given by the intrathecal route but appears to offer no advantage over the sulfonamides. Under special circumstances that warrant its use, i.e., the protection of recruits in the armed services, sulfadiazine is an effective prophylactic also, reducing both the carrier state and the incidence of the disease. With the continued use of sulfonamides for mass prophylaxis, the proportion of resistant strains of Group B has increased, though apparently this has not been true of Group A strains. 17

Bacteriological diagnosis of meningococcal infection. The meningococcus is present in considerable numbers in the spinal fluid in meningitis, and the finding of the characteristic gram-negative intracellular diplo-

cocci in stained smears of the sediment from centrifuged spinal fluid is sufficient to establish a provisional diagnosis. It may be cultured by the direct inoculation of spinal fluid sediment or nasopharyngeal swabs (in the detection of carriers) on infusion base chocolate agar or blood agar. Blood culture is useful early in the disease and in those cases which do not show meningeal symptoms. In asymptomatic carriers meningococci are found in the nasopharynx, and the specimen is taken from this area by swab.60 In any case it is important that the specimen not be allowed to cool below body temperature before inoculation of the warm medium. Like the gonococcus, the meningococcus is oxidase-positive and the oxidase test is especially useful for nasopharyngeal cultures. The isolated meningococci may be identified by fermentation tests and agglutination with polyvalent antiserum. Meningococci may be typed by agglutination, and in recent years the capsular swelling or Quellung reaction, described in the typing of pneumococci, has been used. Chicken antiserum is regarded as superior to rabbit antiserum by many workers. Typing is sometimes desirable in that a rise in the proportionate incidence of Group A usually precedes an epidemic of meningococcal meningitis, and dangerous carriers may be distinguished from relatively ones.4

Pathogenicity for lower animals. usual experimental animals are relatively resistant to the meningococcus upon intraperitoneal or intravenous inoculation. White mice are more susceptible than most other animals, and the injection of sufficient quantities of meningococci will result in death. Rabbits react similarly. Enormous numbers of bacteria must be injected and there is some question as to whether an actual infection is set up; killed meningococci are as effective as the living cells, and it is likely that the observed result is essentially a toxemia. The virulence of meningococcus for the mouse may be greatly increased by suspending the inoculum in mucin, but, as in other instances where it is necessary to use mucin, the relation of the artificial infection to the naturally occurring one is questionable.

Flexner was able to infect rhesus monkeys by the intraspinal inoculation of large amounts of meningococcus cultures, and the disease appeared to be more acute than



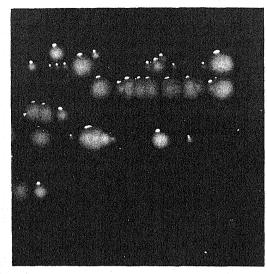


Figure 99. Neisseria catarrhalis. Left, smear from a pure culture. Note the diplococci and elongated forms which have not yet divided. Fuchsin; × 1050. Right, 24-hour culture on blood agar. × 5.

in man. Branham and her colleagues have been able to reproduce meningococcal meningitis in rabbits and in guinea pigs by the intracisternal injection of virulent meningococci. As in man, the experimental disease is both a local meningeal involvement with purulent meningitis and general toxemia. According to Branham it is easier to produce meningitis in guinea pigs than in rabbits. Similarly, a fatal meningoencephalitis may be produced in mice by intracerebral inoculation, though not by other routes. It is of interest to note that the developing chick embryo may be infected with meningococci; the 12-day embryos develop septicemia and hemorrhagic lesions simulating fulminating meningococcus septicemia in man.

Immunity. Infection with the meningococcus leads to the development of demonstrable antibodies, and patient's serum may agglutinate to a titer of 1:50. The development of agglutinins is irregular, or possibly appears so because of the antigenic instability of meningococci, and cannot be depended upon for diagnosis. Complement-fixing antibodies are produced also but too late in the disease (eight to 14 days) to be of diagnostic utility.

The injection of large number of meningococci into horses induces the formation of agglutinins, opsonins, and amboceptors. Antiserum is a reasonably effective therapeutic agent, and reduces the case fatality rate by about half, but is no longer used now that effective chemotherapeutic drugs are available.

Prophylactic vaccination with suspensions of meningococci has been attempted by a number of workers, but the procedure is difficult to evaluate. In general, there appears to be no consistent and unequivocal evidence of a significant degree of protection conferred by active immunization procedures.

Other Gram-negative Diplococci

In addition to the gonococcus and the meningococcus, two other species of non-pigmented gram-negative diplococci are generally recognized.

Neisseria catarrhalis is found commonly in the nasopharynx of healthy individuals as well as of persons suffering from colds and other respiratory infections. The cells as a rule are somewhat smaller than those of the meningococcus. Growth occurs on ordinary nutrient agar much more readily than in the meningococcus, and the colonies are generally thicker and more opaque. Dextrose and other sugars are not fer-

mented. Different strains vary in their pathogenicity for animals, but many strains are fully as pathogenic as meningococci for white mice. In man they appear at times to produce catarrhal inflammation, and sometimes pneumonia and meningitis have been reported. This was relatively common during the 1918 influenza epidemic in some localities.

Neisseria sicca is a small gram-negative coccus found on the mucous membranes of the respiratory tract. It grows at room temperature as well as at 37° C., forms white, firm, dry, adherent colonies, and ferments sucrose, lactose, and maltose. Although not ordinarily regarded as pathogenic, it has been found as apparently the causal agent in a case of kidney infection and has been found in the blood stream of patients ill with clinical endocarditis. This species as well as certain of the pigmented forms is found in an appreciable portion of cases of nongonococcal urethritis,30 but its pathogenic potentialities under these circumstances are not completely clear.

The pigmented forms. Gram-negative diplococci which form a pale greenish yellow pigment often best observed by transmitted light may be found in the upper respiratory tract of man. Although generally considered to be nonpathogenic, they are occasionally found in disease; for example N. perflava and N. flava have been found in meningitis^{36, 62} and endocarditis.⁴⁰ These pigmented forms are differentiated from one another on the basis of fermentations.

The anaerobic species. The obligate anaerobic gram-negative cocci are smaller than Neisseria, 0.3 to 0.4 μ in diameter, and occur in masses and short chains as well as in pairs. These forms appear to be nonpathogenic but occur as parasites in the mouth and respiratory tract and in the intestinal and urogenital tracts as well. They are placed in a separate genus, Veillonella, and divided into two main groups on the basis of gas production in culture mediums. The gas-forming group includes V. parvula, V. alkalescens, and V. discoides, and the nongas-forming group V. reniformis, V. orbiculus, and V. vulvovaginitidis.

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Chapter Twenty

THE ENTERIC BACILLI

The Coliform Bacteria, Friedländer's Bacillus (Pneumobacillus), and Proteus

The gram-negative, nonspore-forming bacilli make up a large group of bacteria which includes intestinal commensals such as the colon bacilli and Proteus; the enteric pathogens such as the typhoid, paratyphoid, and dysentery bacilli; certain saprophytic forms and plant pathogens; and, as more distant relatives, the hemophilic bacteria (Hemophilus); the so-called hemorrhagic septicemia group (Pasteurella); and the causative microorganisms of undulant fever (Brucella).

The largest of these groups, and the subject of this and immediately following chapters, is that of the enteric bacilli, or Enterobacteriaceae. These bacteria are found in the intestinal tract of man and other warm-blooded animals, as commensals of limited pathogenic potentialities associated with diarrheal disease, or, and much less commonly, in infections of the tissues. There are other gram-negative bacilli which are more or less related to the enteric bacilli, but are differentiable from them.

Those grouped in the genus Serratia²⁰ are formally a part of the Enterobacteriaceae by virtue of cultural similarities, but are for the most part free-living saprophytes. Occasionally they are found in association with, and possibly causal relation to, pathological processes.^{26, 34} The most familar of these is the red pigmented Serratia marcescens. The pigment is not apparent in cultures incubated at 37° C., but the colonies are a bright-pink-red at lower temperatures. A majority of the Serratia are not pigmented, and in general these bacteria tend to be

somewhat less active biochemically than many of the enteric bacilli.

The gram-negative bacilli of the Aeromonas group^{24, 25} superficially resemble the enteric bacilli, and in fact certain strains, originally designated C27 paracolon bacilli, contain antigens also found in the Sonne dysentery bacilli. They also resemble Proteus in having polar flagella, and certain of them, like some strains of Proteus, are pathogenic for cold-blooded animals. A considerable number of species have been described from time to time, but these can be put into one or another of three species, viz., Aeromonas hydrophila, which produces red-leg disease of frogs; A. salmonicida, responsible for disease of trout and other fish; and A. shigelloides, antigenically related to dysentery bacilli. Aeromonas has rarely been found in association with disease of warm-blooded animals, including man, and is apparently nonpathogenic for them.

Still other bacteria are closely related to the enteric bacilli, in fact sometimes cannot be distinguished from them with certainty, but are set apart by differences in habitat. Such are Friedländer's bacillus, a common inhabitant of the upper respiratory tract and the causative agent of a small proportion of pneumonias, and the plant pathogens of the genus Erwinia which produce soft rots of vegetables. Similarly, the members of the genera Proteus and Pseudomonas occur as free-living saprophytes as well as intestinal commensals of occasional pathological significance.

CLASSIFICATION OF ENTERIC BACILLI

The differentiation and characterization of the enteric bacilli, or Enterobacteriaceae, is based upon a variety of biochemical and cultural reactions and upon antigenic structure; these bacteria are better known by these conventional criteria than any other group of microorganisms. When the more important of them, the typhoid and paratyphoid bacilli, the colon bacilli, and the dysentery bacilli, were first described and studied, it was not difficult to characterize them with reasonable precision, and they appeared to fall into genera and species in the limited sense in which these concepts seem to be applicable to microorganisms.

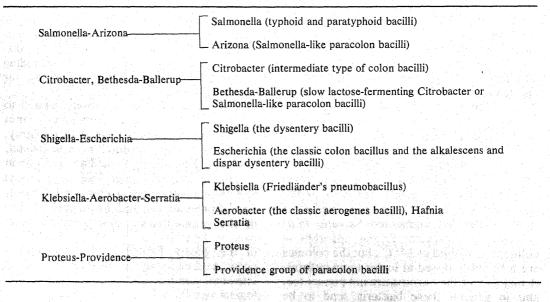
With increasing knowledge, it has become obvious that the enteric bacilli make up a continuous series of forms, showing almost every conceivable combination of differential characteristics. Combinations which have not yet been found in nature have, in some instances, been created in the laboratory by recombination and transduction, with demonstrable fertility groups crossing "genus" lines (Chap. Seven). Sharp distinctions are no longer possible, with different kinds of enteric bacilli merging imperceptibly into one another.

The inadequacy of the usual differential criteria as a basis for a formal classification or taxonomy, as distinct from a differential

key, is clearly evident in this group of bacteria. In fact, it is now becoming generally realized that subdivision of the family Enterobacteriaceae into the conventional tribes, species, and genera cannot be made on other than a genetic basis, which is as yet lacking, and that the species concept in the Linnaean sense is not applicable. At best, the enteric bacilli can be separated into groups, subgroups, and serotypes for purposes of practical expediency,⁴¹ and the construction of an elaborate and ambitious taxonomy of these microorganisms, and by inference possibly others also, is futile.

Nomenclature is another and distinct matter and some workable system is essential. 15, 51 Certain names, originally introduced as generic names, including Salmonella, Shigella, Escherichia, Klebsiella, and Serratia, have gained general acceptance, while others, especially certain species designations, have not. A practical basis of a nomenclature is usage, and therefore familiarity, rather than the priority demanded by the purist, for the latter has often resulted in the creation of new and unfamiliar genera and species which correspond neither with common international practice nor the great bulk of research literature, past and current. Here nomenclature in the controversial area of the enteric bacilli will be based on usage, and names are regarded as no more than useful handles without phylogenetic and taxonomic significance.

Groups and Subgroups of Enterobacteriaceae^{5, 21}



Biochemical Reactions of Enteric Bacilli

	NT	T KLIGLER'S		DEXTROSE	LACTOSE	SUCROSE	MANNITE	SORBITOL	RAFFINOSE	×	GELATIN	MOTILITY	UREASE	OXIDASE	То	METHYL RED	VOGES-PROSKAUER	CITRATE	LYSINE DECARBOXYLASE	LYSINE DEAMINASE	ORNITHINE DECARBOXYLASE	ARGININE DIHYDROLASE
	SLANT	BUTT	H ₂ S	DEX	LAC	suc	MA	SOR	RAF	MILK	GEL	МОТ	URE	OXII	INDOL	MET	vog	CITE	LYS	LYS	ORN	ARG
Escherichia coli Aerobacter aerogenes Aerobacter cloacae Klebsiella pneumoniae	A A A	G G G		G G G G	V V + +	V + +	+ + + +	+ + + +	V + + +	A(C) A(CP) A(CP) A(C)	- +	+ + + -	_	_	+	+	- + + +	1+++	V + - +	_	V + +	V - +
Aeromonas hydrophila (most typical at 30° C.)	K	G	-	G	_	+	+	+			+	+		+	+		+	+	_			+
Serratia marcescens Citrobacter Arizona Hafnia (reactions at 37°C.)	V V V	A G G	- + + w	A G G	V V V	+ V - V	++++	+++-		AP A() A ak	+ - L -	+ + V		_	_	- + + +	+ - V	+ + V	+ - + +		+ V + +	V L
Edwardsiella (Asakusa, Bartholemew)	K	G	+	G		-	-,				_	+			+	+	-	_	+	_		
Salmonella typhi Salmonella paratyphi A Salmonella paratyphi B (S. schottmülleri)	K K K	G	+ - +	A G G			++++	+ + +	+ - V	ak ak ak	_	+ + +	_		-	++++++	_	+	+ - +		- + +	+L +L +L
Salmonella paratyphi C (S. hirschfeldii)	K	G	+	G			+	+		ak	-	+	<u>ئى</u>	_	-	+		+	+	_	+	+L
Salmonella typhimurium	K	G	+	G	_	-	+	+		ak		+			_	+	_	+	+		+	+L
Shigella dysenteriae Serologic group A	K	A	,	A	-		. 7	V	_	_	_	_		-	V	+	-	-	-			. —
Shigella flexneri Serologic group B	K			A	.'		+	V	V		-		-		V	+		-	-			
Shigella boydii Serologic group C Shigella sonnei	K		_	A			+	V	v		_	7 			V	+	· -		_			-L
Serologic group D		A		Α	. :		T.		Υ						-	Т					T	
Vibrio comma	K	A		A	_	+	+				+	+		+	+	+		+	+	_		
Proteus vulgaris Proteus mirabilis Proteus morgani Proteus rettgeri Proteus inconstans (Providence)	K K K K	g g g A g	+ +	g g g A		+ L L L	+			KP KP ak ak ak	++	+ + + + +	++++	- - w	+ + + +	+ + + + +		V V + +	ł	+ + + +	++	
Pseudomonas aeruginosa Mima polymorpha Herellea Moraxella,species	K K K	<u>-</u>		a A -			_			K - V -(P)	+ V V +	+		+ V - +				+++++++++++++++++++++++++++++++++++++++				

Reactions may vary with medium, techniques, and time and temperature of incubation, as well as with strains.

A = acid

a = weak acid

ak = acid changing to alkaline

C = coagulation
G = acid and gas

g = acid and slight or irregular gas

K = alkalineL = late

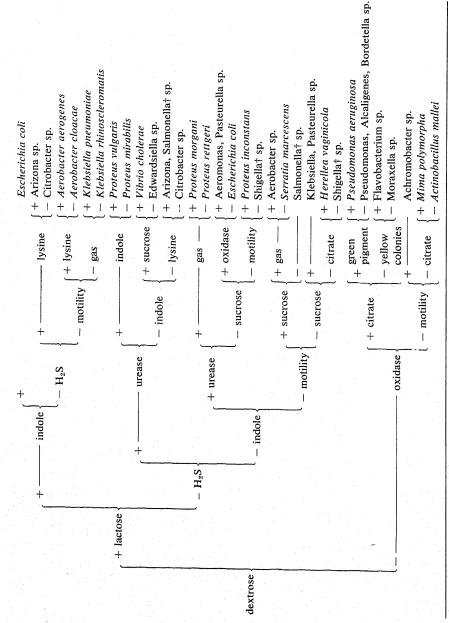
P = peptonization V = variable

w = weak

+ = fermentation or other positive reaction

- = no reaction

Physiological Differentiation of the Gram-Negative Bacilli*



*Prepared by Dr. R. S. Benham and Miss Isabelle Havens. †Differentiated immunologically.

Biochemical differentiation.^{9, 14} A useful primary differentiation can be made on the basis of the lactose fermentation which is roughly correlated with pathogenicity; the coliform bacteria ferment this sugar rapidly with the formation of acid and gas in 24 hours, while the bacteria of the other groups, essentially pathogens (Shigella, Salmonella), do not ferment it. The distinction is not absolute, since both the para-colon bacilli and certain of the dysentery bacilli are slow lactose fermenters, but is sufficiently marked to have considerable practical value.

Similarly, the dysentery bacilli, or Shigellas, divide into two groups on the basis of the fermentation of mannitol and are anaerogenic, that is to say, do not produce gas from fermented carbohydrates. While

the Salmonella group in general produces gaseous fermentations, the typhoid bacillus and Salmonella gallinarum are typically anaerogenic, and anaerogenic strains of other Salmonellas are occasionally met with. The general biochemical groups are idicated in the accompanying table of biochemical reactions.

The forms occurring in specimens from pathological processes in man are separable by systemic application of these biochemical characteristics, both as in the composition of mediums made selective by the inclusion of bile salts and differential by sugars and an indicator, and by specific biochemical tests. The practical application of these differential methods is illustrated in the accompanying scheme.

The Coliform Bacilli

The coliform bacilli were described by Escherich in 1886 as Bacterium coli commune and Bact. lactis aerogenes. It was soon evident that the former occurs as two fermentative types, Bact. coli communior, which ferments sucrose, and Bact. coli communis (or commune), which does not. Still another type, Bact. coli anaerogenes, ferments sugars without the production of gas. The type characterized by a ready mutation with respect to β -galactosidase, *Bact*. coli mutabile, has been discussed elsewhere (Chap. Seven). The generic names Escherichia, Aerobacter, etc. (see below), are now generally used. The microorganism described by Friedländer in 1883 as a causative agent of pneumonia, and known as Friedländer's bacillus or pneumobacillus, is closely related to the coliform bacilli. In addition to these, coliform bacilli characterized by a delayed or late (five to eight days) fermentation of lactose long occupied an anomalous position and were eventually called paracolon bacilli.

These forms are widely distributed and ubiquitous in the sense that they are universally found in the intestinal tract of man and many of the higher animals. Some varieties appear to occur free in nature, but it is probable that these represent contamination, although possibly distant, and these bacteria are primarily parasites of animals.

Morphology and staining. The coliform bacilli exhibit considerable variation in their

morphology. The usual dimensions observed in stained preparations from cultures upon nutrient agar or gelatin range from 2 μ to 4 μ in length and from 0.4 μ to 0.7 μ in breadth. Very short, oval and coccus-like forms are often found and usually predominate when the bacillus is observed directly in the normal animal. The bacilli are occasionally observed in pairs or short chains. Some varieties are encapsulated, particularly those found in pathological conditions. Motility is variable, although the most typical strains are motile by peritrichous flagella. Spores are not formed.

Colonies upon nutrient gelatin are more consistent and characteristic in appearance than those upon agar mediums. They are opaque to partially translucent, smooth, moist, and homogenous in consistency, with entire to undulating edge, and exhibit the maple-leaf appearance common to many of the enteric bacteria. Colonial morphology is somewhat variable upon nutrient agar. Typical colonies are opaque and grayish white, and there may be a tendency to become a light yellowish brown upon continued incubation. Pigmented varieties are occasionally observed. In certain differential mediums the colonies of coliform bacilli may assume other characteristics typical under the special circumstances. Upon Endo's medium, for example, the colonies are, of course, red, but in addition take on a curious metallic sheen that is highly characteristic when viewed by reflected light. Some varieties are β -hemolytic on blood agar; such hemolytic strains occur with much greater frequency in pathological processes than in the normal intestine.

Coliform bacilli stain readily with the ordinary aniline dyes and are gram-negative. Flagella may be demonstrated by special staining methods.

Physiology. The coliform bacilli are facultative anaerobes, growing equally well under aerobic or completely anaerobic conditions. They grow luxuriantly upon the ordinary nutrient mediums and may be cultivated in synthetic solutions containing an ammonium salt and an organic source of carbon such as glucose. Growth occurs over a temperature range of 10° to 46° C., there is good growth from 20° to 40° C., and the optimum is 37° C.

Milk is curdled with an acid reaction usually within 48 hours. Gelatin liquefaction, hydrogen sulfide production, and the formation of indol vary from one kind of coliform bacilli to another and have differential value.

Various sugars are actively fermented with the production of acid and gas. The ability to ferment a wide variety of sugars and other polyhydric alcohols is variable, and some of the fermentations are used for differentiating the various kinds of coliform bacilli. The greater part of the acid produced is lactic acid; smaller quantities of formic and acetic acids are formed, together with ethyl alcohol. Succinic acid is also found in variable but small amounts.

The coliform bacilli are ordinarily resistant to deleterious influences, being, as a rule, neither as resistant as staphylococci nor as susceptible as the more delicate bacteria. Most strains are killed by exposure to 60° C. for 30 minutes, but occasionally more resistant varieties are encountered. In common with the other gram-negative bacteria, they are considerably less susceptible to the bacteriostatic action of dyes than are the gram-positive microorganisms, and selective mediums containing dyes are commonly used in the primary isolation of the enteric bacteria. The ability of the coliform bacilli to grow in the presence of bile is likewise made use of in selective mediums (MacConkey's broth) in the bacteriological examination of water.

Variation. The existence of two colony types of coliform bacilli has long been known, the one the flat, maple-leaf form,

and the other a smaller, raised, round, moist type. The colon bacillus dissociates into rough and smooth colonial types, the S form giving rise to smooth, round, domed, shiny, translucent colonies, while the R colony type is characterized by an irregular dull surface, jagged outline, and opacity. It has been suggested that the R form is the more virulent.

Practically all kinds of microbial variation are not only known to occur among the coliform bacilli, but these have been by far the most commonly used bacteria for such experimental purposes. The K12 strain, for example, was used in the first recombination experiments, the *mutabile* strains provided one of the first and most thoroughly studied examples of mutation, and enzyme adaptation studies have been carried out to a considerable extent with these bacteria.

Toxins. As in the case of many other gram-negative bacteria, the cell substance is toxic to experimental animals upon parenteral inoculation. This endotoxin, presumably occurring as a lipid-polysaccharide-polypeptide complex, is separable as a toxic lipopolysaccharide, and is generally considered to be the prototype of bacterial endotoxins (Chap. Nine). A common antigen, present in very many if not all Enterobacteriaceae has O antigen properties, but apparently has little or no endotoxin activity. 43

A number of strains are hemolytic, as noted above. Two kinds of hemolysins are found. 46,56 One, designated α -hemolysin, is found in liquid culture supernatants, is separable from the cells by filtration, and is thermolabile and lethal for experimental animals. The other, β -hemolysin, is closely associated with the cells. Both lyse a wide variety of erythrocytes. These hemolysins do not appear to be associated with the pathogenesis of infections with hemolytic strains. 57

PHYSIOLOGICAL DIFFERENTIATION OF COLIFORM BACILLI

The coliform bacilli are separated into three groups on the basis of four biochemical reactions, and one of the groups further split using motility and gelatin liquefaction as criteria. The four biochemical reactions are the formation of indol from tryptophan, the methyl red test, the Voges-Proskauer

(V-P) reaction,^{7,59} and the ability to utilize citrate as a sole source of carbon.

The methyl red test is a determination of the pH of a dextrose broth culture after two to four days' incubation. The indicator is added to the incubated culture, and the test is said to be positive when the accumulated acidity is sufficient to turn the indicator red, and negative when the indicator remains yellow.

The Voges-Proskauer reaction is a qualitative test for the presence of acetyl-methylcarbinol among the later end products of glucose fermentation (Chap. Five). After two to four days' growth in glucose-peptone water medium, 5 ml. of a 10 per cent solution of KOH is added. On standing in the presence of alkali, the acetyl-methylcarbinol is oxidized to diacetyl which in turn reacts with some constituent of the peptone to give a pink color. When acetyl-methylcarbinol is produced, the bacterial strain is said to be V-P positive, and when it is not, V-P negative.

These four tests are fixed in order by the mnemonic "Imvic" or "IMViC" and are known as the imvic reactions. While there are 16 possible combinations of these reactions and, in fact, coliform strains giving each of these have been found, three groups are differentiated as indicated in the accompanying table. The classic colon bacillus, Escherichia (Bacterium) coli, is ++--, and the Aerobacter (Cloaca)-Klebsiella group is --++. Only one intermediate group, -+-+, is recognized; the bacteria of this group have been called E. freundii and are now designated Citrobacter freundii. Strains showing this combination of the imvic reactions are also called intermediate strains, or E. coli intermedium, which has no formal status. Other combinations have no formal name and designated "irregular." The relative distribution of these coliform types is shown in the accompanying table.

A number of other tests of biochemical activity are valuable in differentiating the coliforms from one another and from other enteric bacilli. These include the ability to grow in the presence of 1:13,000 KCN, the deamination of phenylalanine to phenylpyruvic acid, the utilization of sodium malonate, and decarboxylase activity with respect to arginine, lysine and ornithine. 18, 47 The usefulness of such tests varies from one group to another; for example, the utilization of malonate is useful in differentiation within the Klebsiella-Aerobacter-Serratia group, and the decarboxlyase reactions in the Aerobacter, Proteus, and Providence groups as well as in enteric bacilli other than coliforms. The utilization of the isomers of tartaric acid, used in the characterization

Percentage Distribution of Coliform Types*

SOURCE	COLI	AER.	INT.	IRR.	TOTAL
Milk	28.8	49.5	20.3	1.4	2224
Water	51.6	28.6	18.5	1.3	9496
Soil	23.8	54.3	18.8	3.1	1330
Grains	17.9	73.8	7.3	1.0	587
Feces	87.9	5.2	6.8	0.1	3974

*As compiled from various authors. In many instances the strains studied were not isolated at random; the percentages are, therefore, weighted, and great significance cannot be attached to them.

of Salmonella species prior to the extensive development of serological typing, has been revived,⁴² and applied more broadly. The physiological character of the coliforms has been compiled and summarized.⁵⁰

The Aerobacter, or Cloaca, group is, as indicated above, broken down into two parts, Aerobacter (Cloaca) and Klebsiella. The former is widely known as Aerobacter aerogenes, corresponding to Escherich's Bact. lactis aerogenes. By definition, A. aerogenes is motile and fails to liquefy gelatin, while Klebsiella is nonmotile and liquefies gelatin slowly. The combination of properties represented by A. aerogenes is, in fact, extremely rare, and most strains which are motile also liquefy gelatin and are, again by definition, A. (Cloaca) cloacae, in the older literature Bact. cloacae. Thus the name A. aerogenes, widely used for coliform strains in genetic and physiological studies and in water bacteriology (Chap. Eleven), tends to drop out of existence. It has been suggested³⁶ that this situation be resolved by creation of a genus Enterobacter to include Aerobacter as two differentiable kinds, cloaceae and aerogenes, leaving Klebsiella to include the nonmotile forms.

In summary the coliform bacilli are separated into the following kinds: Escherichia coli, Citrobacter (Escherichia) freundii, the rare form Aerobacter aerogenes, Aerobacter (Cloaca) cloacae, and Klebsiella pneumoniae, the last Friedländer's bacillus.

THE IMMUNOLOGICAL RELATIONSHIPS OF THE COLIFORM BACILLI

The antigenic structure of the colon bacilli has been elucidated in great detail, and the coliforms may be identified as serotypes with a high degree of precision. 17, 29, 38 There appear to be three kinds of antigens present, viz.:

(1) Heat-stable O antigens, of which more than 100 have been found. Of these, 25 occur with sufficient frequency for diagnostic use with most strains of coliform bacteria.

(2) Somatic surface antigens, designated as "envelope" antigens or K antigens. These function as "blocking antigens" in that their presence interferes with agglutination with O antiserums. Three kinds of

K antigens have been described:

(a) Those which are designated L antigens are thermolabile, and the O agglutinability of bacterial suspensions is restored by boiling. L antiserums may be prepared by absorption of LO serums with the homologous O antigen, viz., boiled bacteria. The colonies of strains containing L antigens are somewhat more opaque than those which do not contain them. About 24 L antigens have been described.

(b) The component of the K antigen designated the A antigen is present in encapsulated coliform bacilli, is a specific polysaccharide, and bacteria containing it give a Quellung reaction in antiserum. It differs from the L antigen in that it is thermostable. So-called N variants lacking the antigen are found in translucent areas at the edge of the large, dense, and relatively opaque colony. About 20 A antigens have been found.

(c) An antigenic component of the K antigen complex, designated the B antigen, is thermolabile but differs from the L antigen in that the heated antigen can absorb antibody though heated suspensions will not agglutinate in monospecific B antiserum. The B antigen

appears to be relatively rare.

(3) The flagellar or H antigens of the coliform bacilli are often poorly developed. Some 22 components have been found, of which 20 are used for purposes of identification.

Kauffmann has developed a serological classification of coliform bacilli, largely based on the distribution of O, K, and H antigens. In general, about 80 per cent of strains having K antigens contain L antigens, and the other 20 per cent contain A antigen or B antigen. Strains containing K antigen appear in a general way to be the more toxic and more resistant to phagocytosis and the bactericidal action of antibody and are found more frequently in pathological material than in feces.

Some of the coliform bacteria are immunologically related to some of the other gram-negative bacilli, such as the plant pathogens, Friedländer's bacillus, Salmonella, Shigella,²³ and Pseudomonas,⁶⁸ while others appear to be related to certain of the pneumococcus types.

The interesting observation has been made⁵⁵ that while most of the colon bacilli harbored by a given individual may be immunologically identical, or nearly so, individuals show a continuous succession of types, each predominating for a few weeks or months and then being replaced by a fresh type, or serotypes may persist for extended periods. Such changes are apparently not associated with host antibody response.⁵² It was noted also that immunologically identical types were sometimes biochemically different.

Pathogenicity for man. The pathogenicity of the colon bacilli in their natural habitat. the intestinal tract, is ordinarily very slight. These bacteria are associated with institutional outbreaks of infant diarrhea, and a number of serotypes are found, and are probably causally related to the disease. 16, 19 Two were differentiated relatively early and have been given a number of designations. One of these has been called Bact, coli neapolitanum, E. coli D433, E. coli α-type, Bact. coli Bray or BGT, and E. Coli O Group 111. The other is E. coli β -type or E. coli O Group 55. These O serological groups, usually designated O55 and O111, are those arbitrarily numbered by Kauffmann, and provide precise identification. E. coli O55 is immunologically related to the pathogenic paracolon bacilli of the Arizona group, and E. coli O111 to the Salmonella antigen XXXV.40

A considerable number of other O serotypes have now been associated with diarrheal disease, including O26, O44, O86, O112, O119, O124, O125, O126, O127, and O128. Compilation of available data²⁸ has shown that the serotypes, more precisely defined by inclusion of additional antigens, most often associated with diarrheal disease in this country are: 055:B5:-;055:B5:H6; O55:B5;H7; O111:B4-; O111:B4:H12; and O127:B8:-. Such complete serotyping is necessary if accurate data are to be obtained.

These serotypes occur for the most part in man, and enteropathogenicity has been demonstrated in human volunteers. ⁶¹ Enteropathogenic strains also give the rabbit gut reaction, ^{63, 66} in which ligated loops of the lower small bowel are infected; this disease model was devised by De and his associates in connection with cholera and has been used extensively in the study of that disease (Chap. Twenty-three). It has been observed

KLEBSIELLA 487

too that positive reactions, manifested as accumulation of intralumenal fluid with marked distention of the loop, are also produced by killed enteropathogenic coliform strains.⁶²

The presence of these serotypes is not necessarily associated with diarrheal disease; *i.e.*, they are not invariably enteropathogenic. In one study⁵⁸ of children in the 0-2 age group, for example, the asymptomatic carrier rate was 5.6 per cent. Of those infected 41 per cent showed no symptoms, and the proportion increased with age.

The causative agents of infant diarrhea differ in various parts of the world and generally reflect economic and public health conditions. In backward countries with a high infant mortality rate due to diarrheal disease, the causative microorganisms are most commonly the Flexner and Shiga dysentery bacilli (Chap. Twenty-two), the common causes of adult dysentery. In advanced countries the dysentery bacilli, and to a considerable extent Salmonella, are less common causes of infant diarrhea, and are replaced by coliforms.⁵³ The picture in this country is similar to, for example, that observed in England⁶⁰ and France.⁴⁵

The laboratory diagnosis of diarrheal disease of coliform etiology has been systematized⁵⁴ and, as indicated above, involves detailed serotyping. A rapid presumptive identification of enteropathogenic coliforms in rectal swabs, using the fluorescent antibody technique, has been worked out in detail^{4, 64} and appears to be very promising.

Outside the intestinal tract, the colon bacilli have definite pathogenic potentialities. The urinary tract is probably the most fre-

quently invaded, and the majority of cases of cystitis are of coliform etiology. In such infections the bacteria are usually present in the urine in numbers of 100,000 or more per milliliter; 20,000 or less probably represents contamination. The colon bacilli may also play a part in the formation of gallstones; the bacillus is frequently found in the core of gallstones and, in culture, can precipitate cholesterol and other biliary constituents. The injection of colon bacilli into the healthy urinary bladder or gallbladder does not produce infection in experimental animals, but infection occurs if the bile duct or urethra is obstructed. Local infections such as abscesses, conjunctivitis, and the like, have been observed but are not common. E. coli septicemia is very rare but may occur as an agonal invasion in acute infective processes. A hemorrhagic septicemia caused by E. coli sometimes occurs in newborn infants and is known as Winckel's disease.

Pathogenicity for lower animals. E. coli is of low pathogenicity for laboratory animals. Two milliliters of a broth culture injected intraperitoneally will kill a guinea pig within a few days, and killed bacilli are very nearly as effective. Spontaneous infection of lower animals is not common. The diarrhea of young calves known as scours has been attributed to E. coli septicemia.³² The colon bacillus is also thought to be a factor in the causation of diarrhea of foals and young pigeons. Local infection may occur; in one series of 286 cases of bovine mastitis, 10 were apparently due to members of the colon-aerogenes group, of which three were typical E. coli.

Friedländer's Bacillus (Klebsiella pneumoniae)

The microorganism described by Friedländer as an etiological agent of pneumonia is, as indicated above, nonmotile and, as found in the respiratory tract, usually heavily encapsulated. Motile strains occur in such pneumonias, however, and strictly speaking they are not *Klebsiella pneumoniae*, but the distinction is not relevant to the disease produced. Occasional strains are pleomorphic in culture. Friedländer's bacillus is readily isolated from sputum from cases of

the pneumonia caused by it by culture on blood agar. The growth is heavy and viscous, and the colonies unusually large. For practical purposes, this kind of growth and demonstration of the gram-negative, thick, ovoid rods, often occurring in pairs, suffices for identification.

As indicated earlier, these bacteria are extremely closely related to the aerogenes type of coliform. Differentiation may be exceedingly difficult^{30, 49} if not practically

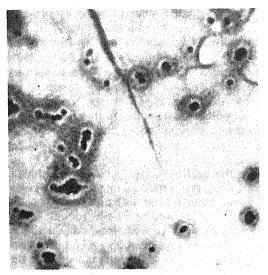


Figure 100. Klebsiella pneumoniae (Friedländer's bacillus) in pure culture on blood agar, showing capsules. Crystal violet; × 1200.

impossible at times, and is irrelevant to the bacteriological diagnosis of pneumonia. Detailed examination of strains allows differentiation within Klebsiella into K. pneumoniae, K. ozaenae and K. rhinoscleromatis, the last two being associated with a type of chronic upper respiratory tract infection, together with certain other types given species names. 6, 39

Immunological types. This group of bacteria, including both motile and non-motile forms, contains three kinds of antigens, designated O, A, and K, and analogous to those of *E. coli*. The bacteria are typed on the basis of the capsular antigen, and a total of 57 capsular types have been described. The Quellung reaction does not occur in specific antiserum, but a precipitin reaction takes place at the periphery of the capsule, making the margin highly refractile. There appears to be no variation in virulence associated with capsular type.

Pathogenicity. Microorganisms of this group are found associated with various kinds of upper respiratory disease in man, and in most instances are probably secondary invaders, as in the nasopharynx in persons having cronic sinusitis or chronic lung infections such as bronchiectasis.

Pneumonia due to Friedländer's bacillus makes up 0.5 to 4.0 per cent of all pneumonia, but the case fatality rate is high, 90 per cent or more in untreated cases. The microorganism has a greater tendency to

produce necrotic lesions than pneumococcus, and the infection contrasts with pneumococcal pneumonia in that while it is similar in its very early stages with a spreading zone of infected edema around an older area of dense exudate, as the infection progresses the alveolar walls become involved and disintegrate, and multiple areas of purulent exudate become necrotic. Thus the pathological response is necrosis of the lung parenchyma and subsequent healing with fibrosis, commonly accompanied by abscess formation, cavitation, or bronchiectasis. The sulfonamides and broadspectrum antibiotics, but not penicillin, are reasonably effective, and according to some workers are more effective in combination:

Probably antimicrobial treatment instituted very early would result in complete recovery, but the infected individual is usually not hospitalized until the second day, and another day is wasted with penicillin therapy. In the drug-arrested infection, the affected areas become a lesion consisting of one or more cavities lined with necrotic material connected to damaged bronchi, and chemotherapy transforms the acute, often fatal, condition to a subacute or chronic infection.

Despite chemotherapy the residual lung damage frequently requires surgical drainage and later pulmonary resection. During surgery there is some likelihood of a flare-up

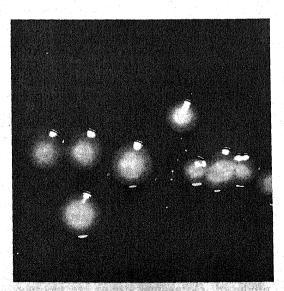


Figure 101. Colonies of Friedländer's bacillus on blood agar. Note the large size and mucoid appearance.

of infection in the contralateral lung due to persistence and drug resistance of the infecting microorganism.^{37, 69}

Klebsiella has also been associated from time to time with suppurative conditions in various parts of the body, such as liver abscess and meningitis, 65 and rarely has been found to invade the blood stream to produce septicemia. Infection may also occur spontaneously in lower animals, and these bacteria have been found to be the etiological agents of epidemic respiratory infection in mice, a paralytic disease of moose, and metritis of mares.

The Paracolon Bacilli

The term paracolon bacillus is applied to enteric bacilli similar to the colon-aerogenes group, but distinguished from it by a delayed (five to 21 days) fermentation of lactose. On the basis of the IMViC reactions, these slow lactose fermenters range from a typical coli type to a typical aerogenes type. Certain biochemical types of paracolon bacilli closely resemble the Salmonellas and, especially when the lactose fermentation is long delayed, may present a practical problem in reasonably rapid identification.

Study of the serological character of some of these forms, especially those that are found in apparent etiological relationship to enteric disease, has shown that they are related on the one hand to coliform bacilli and on the other to Salmonella, and some antigenic components are shared with certain of the dysentery bacilli. Still others are more closely related to Proteus. Their division into groups is more a matter of convenience for purposes of study than a recognition of sharply defined differences among them. A number of such groups are generally recognized;8 a rough approximation of their relationship to one another and to other enteric bacilli, arrived at only by disregard of some apparently minor resemblances, is shown in the accompanying diagram.

The Bethesda-Ballerup group. 31, 67 The paracolon bacilli in this group closely resemble the Salmonellas in the usual biochemical characteristics except that they ordinarily, but not invariably, ferment lactose and sucrose, and often salicin, on prolonged incubation. They were originally regarded as two separate types, one of which was described as Salmonella ballerup though it was immunologically related to the other Salmonella types only through its content of Vi antigen.

The first of the Bethesda group of para-

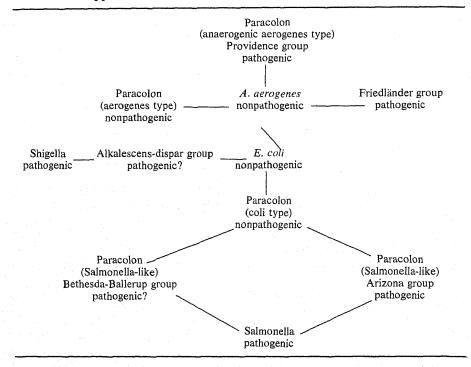
colon bacilli were originally isolated in Bethesda, Maryland, but subsequently they have been found widely scattered over the country. Many of the Ballerup strains are late lactose and/or sucrose fermenters and are now regarded as paracolons, and have been merged with the Bethesda group since the two are closely related antigenically. So far 32 O groups and 74 H antigens are recognized and occur in various combinations to give a multiplicity of serological types. These bacteria have been isolated with some frequency in association with enteric disease, and at least some strains are possible pathogens. They are often encountered in diagnostic work and are not rapidly separable from Salmonella by biochemical tests; pooled and polyvalent antiserums facilitate their recognition.

The Arizona group. 12, 22 These bacteria were first described as Salmonella found in reptiles and resemble the Salmonellas closely except that most strains ferment lactose within two weeks. They are closely related serologically to Salmonella; many of the O antigens are identical with those of Salmonella, and the H antigens are closely similar. These bacteria fall into 76 O groups, and 75 serological types have been described. It appears to be definitely established that this group of paracolon bacilli is regularly pathogenic, not only for coldblooded animals, but also for fowls and man, and in the last two produces infections as severe and fatal as do the Salmonellas.

The Providence group.¹³ This group is made up of paracolon bacilli of an aerogenes type (citrate-positive) that are anaerogenic, *i.e.*, produce little or no gas from glucose. They were originally described as the 29911 group but are now known as the Providence*

^{*}They were originally described and have been studied most intensively by Stuart and his co-workers at Brown University in Providence, Rhode Island,

Approximate Interrelationships of the Enteric Bacilli



group. They are closely related to Proteus, so much so that it has been suggested that they be considered as a species of Proteus, *Pr. inconstans*. They have been associated with institutional outbreaks of diarrhea

among infants and may be regarded as possible pathogens. Their serology is as yet incompletely worked out, and this has hampered their identification in association with enteric disease.

Proteus 13

These microorganisms were originally described by Hauser as an independent genus containing three species-Pr. vulgaris, Pr. mirabilis, and Pr. zenkeri. It is now generally agreed that the last is not closely related to the typical members of the group (it is gram-positive), and it is now placed in a separate genus as Kurthia zenkeri. As indicated earlier, Proteus is included under the Enterobacteriaceae and is related to but nevertheless distinct from the other enteric bacilli. It is found with some frequency in normal feces and often increases proportionately during or immediately after attacks of diarrheal disease caused by other organisms. It is one of the most common bacteria in soil and water containing decaying organic matter of animal origin and usually occurs in large numbers in sewage; it is perhaps to be identified with the "bacterium of putre-faction" or *Bact. termo* of early writers. Like the enteric pathogens it does not ferment lactose, and it resembles them in its growth on differential and selective mediums. It may be confused with Salmonella because of its motility and gas formation during carbohydrate fermentation, but is distinguished by its ability to hydrolyze urea.

Morphology and staining. In general, these bacteria appear as straight or slightly curved rods 1 to 2.5 μ in length and 0.4 to 0.6 μ in breadth, frequently in end-to-end pairs and short chains. Ovoid forms are common, however, and long, curved, filamentous cells predominate in actively

swarming cultures. Proteus is actively motile by peritrichous flagella and forms neither capsules nor spores.

The phenomenon of "swarming" exhibited by these bacilli is a consequence of their active motility. On the surface of agar mediums the colonies do not remain compact and discrete; the growth spreads rapidly over the entire surface available as a thin. scarcely visible bluish film. Microscopic observation shows that the bacilli break away from the edge of the growth and migrate or "swarm" over the surface of the medium, thus giving rise to the thin film of growth. This property is a source of considerable inconvenience in the isolation of bacteria other than Proteus from mixed cultures in which it is present; the inclusion of 0.01 per cent sodium azide inhibits the growth of this and other gram-negative bacilli but allows the growth of streptococci.

These bacilli stain readily with the usual aniline dyes and are gram-negative.

Physiology. The nutritive requirements of Proteus are simple, and the bacilli grow readily upon the ordinary laboratory mediums. They may be cultivated in synthetic solutions containing ammonium lactate, but nicotinic acid must be supplied. The optimum temperature for growth is 30° to 37° C., though good growth occurs at 20° C. These bacteria are facultative anaerobes, but anaerobic growth is generally very poor.

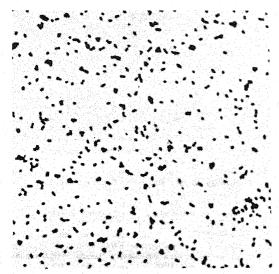


Figure 102. Proteus vulgaris. Smear from a pure culture. Note the coccobacillary form. The occasional occurrence of paired cells is a result of active multiplication. Fuchsin; × 1050.



Figure 103. Proteus vulgaris colony on blood agar. Note the swarming, exhibited as successive waves of growth. (Dack.)

Gelatin is liquefied more or less rapidly, often with a characteristic colony formation with radiating filaments which wander far off into the surrounding medium. The typical Proteus colonies are best formed when the gelatin is soft, as happens when it is kept at a temperature not far below the melting point or is made up with 5 instead of 10 per cent gelatin. Dextrose is fermented with actd and gas production; sucrose and maltose are fermented by some, but not by all, strains; lactose, raffinose, and mannitol are never fermented by the typical Pr. vulgaris. The Voges-Proskauer reaction is negative and the methyl red test positive. Nitrates are reduced. Milk is at first rendered slightly acid, then curdled with alkaline reaction (in about three days), and more or less slowly peptonized. While species of Proteus other than Pr. morgani are actively proteolytic, these bacteria apparently do not play an important part in the anaerobic decomposition of proteins or putrefaction as was once supposed. Under aerobic conditions proteolysis occurs rapidly, i.e., decay or nonputrefactive proteolysis.

At the present time the bacteria comprising the genus Proteus are classified entirely on a biochemical basis.²⁷ As noted above, the group is distinguished by its ability to hydrolyze urea. Four species are recognized: Pr. vulgaris, Pr. mirabilis, Pr. rettgeri, and Pr. morgani; the last is known in the earlier literature as Morgan's bacillus (see below). These are distinguished from one another on the basis of the fermentation of mannitol, maltose, and sucrose and the formation of indol.

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Antigenic structure. Proteus contains both H and O antigens when motile, and the nonmotile, nonswarming strains contain only O antigen. Three colonial types have been observed, and antigenic analysis of the O and H antigens has given 18 O groups in 44 strains, with recurring and crossing of H antigens and some suggestion of phase variation. The ureases of Pr. vulgaris, Pr. mirabilis, and Pr. rettgeri have been found to be serologically indistinguishable, but that of Pr. morgani is antigenically distinct. The ureases of the colonial distinct.

Certain Proteus strains are agglutinated by the serum of patients having typhus fever. These so-called X strains contain an antigen common to the typhus rickettsiae, and the agglutination of these bacilli (the Weil-Felix reaction) is of diagnostic value in typhus fever. In the Proteus strains the antigen is a part of the O antigen, and its specificity is determined by an alkali-stable carbohydrate haptene which is also found in *Rickettsia prowazeki*. The X strains frequently ferment maltose.

Pathogenicity. Proteus, both in mixed and pure cultures, has been found to be associated with a variety of pathological conditions. Infections of the eye and ear, pleuritis and peritonitis and suppurative abscesses in many parts of the body are among the many instances in which an etiological role is highly probable. As a producer of cystitis and pyelonephritis it ranks next to E. coli. In experimental infections of the mouse kidney, Proteus has been found to be considerably more virulent than Pseudomonas or Escherichia.33 There is evidence³ that Proteus urease is nephrotoxic. favoring the intracellular infection of the tubular epithelium and creating an alkalinity in the kidney which leads to necrosis of renal tubular epithelium with the precipitation of MgHPO4 with the formation of stones. Besides its independent pathogenicity, it is found commonly associated with other microorganisms in purulent war wounds and similar processes.

In certain affections of the digestive tract Proteus has been frequently held to be the responsible agent. In diarrheic stools, especially those of infants, it has often been found in large numbers and is regarded by many as a cause of infant diarrhea. The real relation of Proteus to intestinal infection is, however, still obscure. Certain foodpoisoning epidemics have been ascribed to Proteus.

Animal inoculation shows that a great range of virulence exists among the Proteus cultures that have been tested. Freshly isolated strains from pathological sources may produce definite lesions, including abscesses, enlargement of the spleen, and a diarrheic condition. The etiological agent of red-leg disease of frogs, ulcer disease of brook trout, and red sore of pike has been found to be a bacillus closely related to the Proteus group, and it has been classified as Pr. hydrophilus, 44, 48 and is not to be confused with Aeromonas species, which are also pathogenic for cold-blooded animals to produce similar diseases. The cell substance of these bacteria is toxic on parenteral injection and, as in the case of the enteric forms, the toxin appears to be a glucolipid. No difference in toxicity is apparent between strains of pathogenic and nonpathogenic origin.

This bacillus was Morgan's bacillus. isolated by Morgan in 1906 from the stools of infants suffering from summer diarrhea. Although it was first designated as Morgan's No. 1, the other varieties of Morgan's bacilli have long since faded out of general interest and recognition so that the name Morgan's bacillus is usually applied to Morgan's No. 1. It is closely related to the Proteus group, and it is classified as Pr. morgani. This bacillus appears to have played a part in a number of outbreaks of summer diarrhea of infants and has been isolated from paratyphoid-like fevers. It has been found to give rise to spontaneous epidemics of enteritis in mice. Injected intraperitoneally into mice, Morgan's bacillus produces a rapidly fatal infection.

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Chapter Twenty-one

THE ENTERIC BACILLI

The Salmonella Group

The enteric bacilli comprising the large Salmonella group are all pathogenic to a greater or lesser degree and include the typhoid bacillus, the paratyphoid bacilli, and a wide variety of forms whose natural hosts are lower animals, especially rodents and birds.

The infectious nature of typhoid fever was apparent in 1856 to William Budd, who, on the basis of epidemiological evidence, suggested that the disease was transmitted by sewage-contaminated water and that the source of the infectious agent was human feces. The typhoid bacillus was found by Eberth in 1880 in the mesenteric glands and spleen of persons dying from typhoid fever and was cultured in 1884 by Gaffky.

The rodent pathogen, Salmonella enteritidis, was described in 1888 by Gärtner, who isolated it from infected beef responsible for an outbreak of gastroenteritis, and in the older literature this microorganism is known as Gärtner's bacillus.

For a good many years the kinds of microorganisms making up the "typhoid-paratyphoid group" were relatively few and confined largely to those differentiable by cultural reactions. With the development of the techniques of antigenic analysis and their application to these microorganisms in the 1920's, very many serologically differentiable types or kinds were, and continue to be, described until by now the Salmonella group is made up of many hundreds of differentiable types.^{29, 54}

Morphology and staining. These bacilli are gram-negative rods closely resembling and indistinguishable from the coliform bacteria. They stain readily with the usual dyes such as methylene blue and carbol-

fuchsin. No particular arrangement of the cells is apparent on microscopic examination. All species except *Sal. pullorum* and *Sal. gallinarum* are actively motile by means of peritrichous flagella. No capsules are apparent, and spores are not formed.

Physiology. The bacteria of this group have simple nutritional requirements, growing readily on the usual nutrient mediums. In synthetic mediums an ammonium salt and glucose, pyruvate, lactate, etc., are adequate sources of nitrogen and carbon. The great majority of strains do not require bacterial vitamins or amino acids, but some strains of the typhoid bacillus require added tryptophan. The optimum temperature is 37° C., but growth occurs at a reasonable rate at room temperature. They are facultative anaerobes, growing equally well under either aerobic or anaerobic conditions, and some species develop relatively strong reducing tendencies.

The group is characterized biochemically by failure to ferment lactose or salicin and inability to liquefy gelatin or produce indol. There are a very few exceptions to the two last; Sal. eastbourne and some strains of Sal. enteritidis and Sal. panama produce indol, and Sal. daressalaam liquefies gelatin. The evolution of gas commonly accompanies the fermentation of sugars, though anaerogenic strains of Sal. enteritidis, Sal. typhimurium, and Sal. paratyphi C have been reported. Sal. typhi, however, characteristically ferments sugars without the formation of gas.

A great variety of cultural reactions, not only the conventional sugar fermentations, formation of indol from tryptophan, etc., but various kinds of specialized tests, such

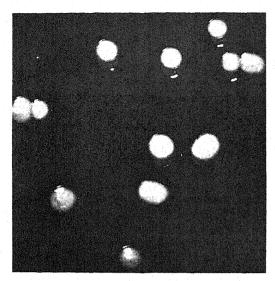


Figure 104. Colonies of Salmonella typhimurium (aertrycke) on nutrient agar, 24-hour culture. × 3.

as the utilization of the tartaric acids and malonate and the amino acid decarboxylase activity, have been useful in the physiological characterization of bacteria of this group.^{26, 28}

Toxins. Like the other enteric bacilli, the Salmonellas do not form exotoxins but all contain endotoxins. These are the polysaccharide-polypeptide-lipid complexes extractable from the intact cells with trichloracetic acid or glycols and are extracted as toxic lipopolysaccharide in 50 per cent

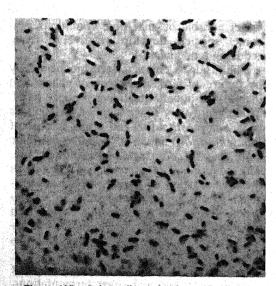


Figure 105. Salmonella typhi. Smear from a pure culture, Sommersby strain. Note the variation in size from coccoid to bacillary forms. Fuchsin; × 1050.

phenol. They appear to occur largely in the cell wall of the bacteria. These endotoxins, together with those of the coliform bacilli, have been taken as prototypes and most of the information available on endotoxins (Chap. Nine) has been derived from them.

The toxicity is nonspecific in the sense that endotoxins from whatever source produce substantially the same reactions on parenteral inoculation. The more obvious responses are fever and alteration in capillary permeability, the former probably contributing to the symptoms of the disease. The endotoxins are antigenic, stimulating the formation of agglutinating, precipitating, and protective antibody activity, but the antitoxic activity of antibody is of a low order. The specificity is that of the contained polysaccharide, and the endotoxins are apparently identical with the heat-stable somatic antigenic complex of the bacteria.⁵²

Another toxic substance, extractable in alcohol and named the Q substance, has been found in typhoid bacilli and other enteric bacilli, but its relation to the endotoxin is uncertain.

Immunological differentiation. As indicated above, the techniques of antigenic analysis have been developed in the study of the Salmonella group, and the antigenic composition of bacteria of this group is, perhaps, better known than that of any other bacteria. Many members of the group are immunologically complex; whether because they are innately so or because they are better known in this respect than most other bacteria is difficult to say.

Flagellar and somatic antigens. Two types of antigens are present in the bacteria of the Salmonella group, one associated with the cell substance and the other with the flagella. Noted by Smith and Reagh in 1903, the first was termed somatic antigen and the second flagellar antigen. These types were later observed by Weil and Felix, who designated them O and H antigens respectively. The O antigens are arbitrarily designated by numerals.

These two types of antigen, each of which may be, and frequently is, represented in a single bacterial strain by more than one component, differ from one another in several respects. The flagellar antigen is the more unstable and is destroyed by boiling and by exposure to alcohol or weak acid; somatic antigen, on the other hand, is stable to boiling, alcohol, and acid. Stock somatic

antigen for immunization purposes may be prepared by heating at 100° C., followed by treatment with alcohol and drying with acetone.21 Cultures on phenol agar (0.1 per cent) of bacteria normally containing both H and O antigens are found to contain only O antigens, the formation of flagellar antigen having been suppressed; H antigen reappears immediately on cultivation on nutrient agar. In the agglutination reaction, bacteria lacking flagellar antigen are characteristically clumped in a finely granular precipitate (O agglutination), while bacteria containing flagellar antigen are agglutinated in a coarse, flocculent precipitate (H agglutination).

The H and O agglutination titer of an antiserum may be determined by the use of H and O antigens in the agglutination test. H antigen is commonly prepared by adding an equal volume of formol (0.6 per cent formalin) saline to an 18- to 24-hour broth culture. In the preparation of somatic antigen the flagellar component is destroyed by treatment with alcohol; the growth from an 18- to 24-hour agar slant culture is emulsified in 1 to 2 ml. of absolute alcohol, heated at 60° C. for one hour, centrifuged, and the sediment suspended in 0.5 to 1 ml. saline. It may be used for slide agglutination or appropriately diluted for macroscopic agglutinin titrations.

These two types of antigen are immunologically independent, and the immunization of an animal with a microorganism containing both results in the production of antibodies to both. There is, however, a marked difference in titer, for the O antibody titer is generally much lower than that of the H antibody; in the writer's laboratory antiserums having H titers of 1:20,000 to 1:50,000 have shown O titers of 1:2000 or less.

SPECIFIC AND NONSPECIFIC FLAGELLAR ANTIGENS. The flagellar antigen is, in turn, of dual nature. One kind, designated as specific flagellar antigen, is individualistic and contributes in no small part to the immunological identity of a given Salmonella species. The other, termed nonspecific flagellar antigen, is made up of a limited number of components which are frequently shared by the various Salmonellas and hence contribute to the immunological relationship between the species of these bacteria. The specific flagellar antigens are arbitrarily designated by lower case letters (the choice

was unfortunate since the more recently discovered antigens are designated z_1 , z_2 , z_3 , etc.), and the nonspecific ones by Arabic numerals. Strains containing both kinds of H antigens are called diphasic, and those containing only either one or the other are called monophasic.

VI ANTIGENS. A somatic antigen, closely similar to, but not identical with, the O antigen complex, and designated Vi antigen, occurs in Sal. typhi, Sal. paratyphi A, Sal. paratyphi C, and Ballerup strains of paracolon bacilli.31 It resembles the O antigens in that it is present in the cell substance of the microorganisms and is extractable with trichloracetic acid but is more labile to heat in the presence of water and to treatment with dilute alkali.5, 48, 83 It is present in practically all strains of the typhoid bacillus on primary isolation, but in only some strains of para A and para C, and has the same immunological specificity in all the microorganisms in which it occurs. It was named Vi antigen, or "virulence antigen," because its presence is believed by some to be associated with virulence, and antibody to it is protective.34

Antigenic structure. The components of the somatic and flagellar antigenic mosaics of bacteria of this group have been worked out in detail by application of the methods of antigenic analysis, *i.e.*, reciprocal agglutinin absorption (Chap. Fourteen). Each of the serological types of Salmonella may be defined in immunological terms as antigenic formulas; representative antigenic formulas are shown in the accompanying table.

This kind of pattern is known as the Kauffmann-White schema.⁴⁶ There is some association between antigenic entities in that they tend to appear in recurring combinations, and there is evidence which suggests that certain of the immunologically separable activities, *i.e.*, by absorption of antibody, may be closely bound or even repre-

Representative Antigenic Formulas

O GROUP	SPECIES	ANTIGENIC FORMULA
D	Sal. typhi	IX, XII, (Vi); d: -
Α	Sal. paratyphi A	I, II, XII: a: -
В	Sal. paratyphi B	I, IV, V, XII: b: 1, 2
C,	Sal. paratyphi C	VI, VII, (Vi): c: 1, 5
$C_{\mathbf{i}}$	Sal. cholerae-suis	VI, VII: c: 1, 5
В	Sal. typhimurium	I. IV, V, XII; i: 1, 2
D	Sal. enteritidis	I, IX, XII: g, m: -

sent differing specificities contained in the same antigen molecule.14 Even with such associations to limit possible combinations of antigens within the same bacterium, a great number of serotypes is possible. New serotypes are continuously described, and from time to time the relevant data are aggregated.⁵⁸ Combinations of antigens, e.g., serotypes, can be produced in the laboratory (see below), and their natural occurrence is perhaps a consequence of conjugation and transduction as well as loss variations. In any case, the relative fluidity of antigenic structures leads to a certain skepticism as to the status of serotypes in classification (see below) although they are extremely useful in practice.72

Such antigenic structures have an illusory precision, illusory in that other antigenic components occur⁸⁴ but are neglected for practical purposes, and also because a single antigen, as designated by a symbol, may in fact be a complex in that there are small variations in it as it occurs in different microorganisms. Such differences, as well as similarities, are demonstrable in O antigen having polysaccharide specificities as chemical differences,⁵² and also in at least some of the flagellar antigens.⁶³

Salmonella typing. Partial or complete identification of such serotypes is carried out by the use of antiserums containing appropriate antibody; monospecific antiserums may be prepared by absorption to contain antibody to a single antigenic component when necessary. The serological identification of Salmonella strains in this way is known as Salmonella typing.

The somatic antigen provides the basis for primary separation of these serotypes into groups. There are 13 such groups in all, the first 12 made up of serotypes having common and identifying O antigens, and the last those serotypes which do not fit in elsewhere. These groups are:

I, II, XII
(I), IV , (X) , XII
VI, VII
VI, VIII
(I), IX , XII
III, X
III, X
III, XIX
XI
(I), XIII, XXIII
(I), VI, XIV, XXV
XVI

About 98 per cent of Salmonella strains isolated fall into the first eight groups, A to E₃ inclusive, and the identifying antigens of these groups are shown in boldface type above, while those antigens which occur in some but not all serotypes or strains of the group are shown in parentheses.

It is, then, a relatively simple matter to assign a strain to one or another of these groups by the use of appropriate antiserums, and identification may be carried considerably further with relatively few additional antiserums to the H antigens. The antigens used to prepare common typing antiserums are shown in the accompanying table. Complete analysis of the antigenic structure of a strain, or identification of the small portion of isolates falling outside these common somatic groups, becomes a specialized procedure.

Classification. The Salmonella group is relatively homogeneous in that these bacteria resemble one another more closely than they resemble other enteric bacilli, although the Arizona and Bethesda-Baller-up groups of paracolon bacilli are "Salmonella-like," and provide connecting links between Salmonella and the coliform bacilli. It is little more than a matter of arbitrary definition as to whether or not the Salmo-

Salmonella Typing Serums

O antiserums H antiserums				
GROUP	ANTISERUM	ANTIGEN	ANTISERUM	ANTIGEN
A	I, II, XII	Sal. paratyphi A	a	Sal. paratyphi A
В	IV, V, XII	Sal, paratyphi B	i i na mingrapi ku b anjasi kiwa	Sal. paratyphi B, phase 1
C _t	VI, VII	Sal. thompson		Sal. cholerae-suis, phase 1
C.	VIII	Sal. virginia	and the second distribution of the	Sal. typhi
D	IX, XII	Sal. gallinarum	i	Sal. typhimurium, phase 1
Е	III, X, XV	Sal. anatum and Sal. newington	1, 2, 3, 5	Sal. thompson, phase 2 and Sal. newport, phase 2

VARIATION 499

nella group should be made larger and more complex by the inclusion within it of, for example, the Arizona group of paracolon bacilli.

Nomenclature within the group is another matter. Certain well-established differentiated by physiological methods prior to the application of antigenic analysis. such as those for which representative antigenic formulas are given, retain names such as Sal. typhi and Sal. enteritidis. As new and additional serotypes have been described, it has been customary to give them place names and implied species status, viz., Sal. newport, Sal. montevideo, Sal. panama. etc. An alternative to the use of a great many such names has been to give these but a single name, such as Sal. enterica, further identified by antigenic group or formula. This has not met with general acceptance, and the multiplicity of serotypes continue to be known by a multiplicity of names. As indicated earlier (Chap. Twenty), the use of such names is not to be taken as indicating that these serotypes are species in the Linnaean sense.

Variation. Possibly because the antigenic structure of the Salmonella group is known in considerable detail, three general types of variation may be differentiated. One of these is a type of fluctuating, completely reversible immunological variation known as phase variation, the second induced variation, and the last S-R dissociation known to occur in practically all bacteria.

Phase variation. The H antigens of certain Salmonella types can be separated into relatively stable components. This may be shown very simply by plating out such a type and carrying out slide agglutination of individual colonies with monospecific antiserum. About half the colonies will be found to contain one kind of antigen and about half the other. Apparently the individual bacterial cells do not contain both kinds of H antigen but are of two kinds in this This immunological character breeds true to only a limited extent, for if a colony is picked and subcultured in broth, platings of successive broth cultures will show a rapid reversion to the 50:50 ratio within a very few transfers. This is considered to be a mutation-back mutation process occurring at a relatively high (of the order of 10^{-3} to 10^{-4}) rate.^{49, 70} This type of immunological variation is called phase variation and the transitory immunological

types were originally called the specific phase, i.e., that characterized by the presence of specific flagellar antigens, and the nonspecific phase, i.e., that in which the nonspecific antigens occurred. Phase variation in one direction only, giving rise to a monophasic strain or mutant, has been observed.⁷³ This division is not as sharp as once thought, however, and now these phases are commonly referred to as phase 1 and phase 2 respectively. The Salmonella types that exist in two immunological phases are termed diphasic, while those that exist in only one phase, which may be either phase 1 or phase 2, are called monophasic. The presence of three phases appears to occur occasionally.50

Types of phase variation. Phase variation is somewhat more complex than this, however, and three types have been described. The first is the specific-nonspecific phase variation in which a specific antigen ordinarily occurring in phase 1 is linked with the nonspecific antigens of phase 2. The second is the so-called α - β phase variation in which an antigen occurring in phase 1 is linked with the antigens e, n, +, in phase 2. Five types of α - β phase variation have been reported. Lastly, there is a type of phase variation which has not been named in which the antigens of both phases are commonly found in phase 1.

O antigen variation. It is not clear whether this type of immunological variation occurs generally with respect to the somatic antigens. A similar variation, known as form variation, does occur, however. Of the three components of XII, designated XII₁, XII₂, and XII₃, XII₂ varies in that it is either strongly or weakly developed.^{24, 45} Antigen VI is likewise subdivided into VI₁ and VI₂ and form variation occurs in the former.²⁰

VW VARIATION. Variation in the Vi antigen content of the typhoid bacillus occurs as a tendency of freshly isolated, Vicontaining strains to lose Vi antigen within a few transplants on culture mediums. Vicontaining strains which presumably contain a maximum amount of this antigen are inagglutinable in O antiserum, the Vi antigen apparently having a masking effect, and are known as V strains. On culture, the V form becomes O-agglutinable, but still contains Vi antigen and absorbs Vi antibody from serum, and is called a VW strain. The VW form then loses its Vi antigen en-

tirely and becomes the W form. The Vi antigen is apparently independent of the other antigenic components of the typhoid bacillus, or those of other bacteria in which it occurs, and its presence or absence is unrelated to the nature of the O and H antigenic complexes.

It is to be emphasized that the immunological changes associated with phase variation are, so far as is known, normal, the bacterial strain seemingly existing in a sort of immunological equilibrium. These variations and the complete but temporary loss of flagellar antigen by cultivation on phenol agar noted above apparently bear no relation to the S-R dissociation, though, as indicated, they may be induced by a method which will induce dissociation, *i.e.*, cultivation in specific antiserum.

Induced variation. Antigenic changes may be produced in the H antigens of a number of Salmonella species by cultivation in the presence of appropriate antiserums. It has been shown that Sal. paratyphi A, a monophasic type stable in phase 1, can be induced to form phase 2 antigens by cultivation in antiserum specific to the phase 1 antigens. Conversely, diphasic types can be stabilized in one phase by cultivation in the presence of antiserum to the antigens of the other phase. By this means Salmonella "species" may be transformed, for example, Sal. simsbury into Sal. senftenberg.27 Furthermore, "new" species may be created from monophasic types by suppression of flagellar antigen through cultivation in the presence of monospecific antiserum, with the appearance of a hitherto unknown flagellar phase. A number of such induced antigens have subsequently been found to occur naturally.18

O antigen specificity is altered in the S-R dissociation as indicated below, but this is presumably unrelated to changes analogous to induced changes in H antigen that have been produced by cultivation in the presence of antiserum in semisolid mediums. By this means changes have been induced in O antigen within groups only; Sal. anatum has been changed to Sal. newington, and the change reversed.

Dissociation. S-R dissociation, similar in all respects to that known in other bacteria, occurs in cultures of these bacilli. The dissociation from smooth to rough is manifested as an alteration in colonial morphology and loss of virulence. The change

is reflected immunologically as a loss of specificity of the somatic antigens; *i.e.*, the rough forms remain motile. The specificity of these antigens is apparently determined by a polysaccharide haptene, and with the disappearance of the haptene the bacteria acquire a new and common immunological character in the somatic antigens, while the flagellar antigens remain unchanged. A mucoid or M phase in colonial morphology has been reported by some workers which is said to be associated with the development of a new immunological specificity.

Phage typing.³² Salmonella strains of the same serotype, and indistinguishable by serological, protective, or biochemical tests, may be subdivided into phage types on the basis of their susceptibility to lysis by different races of bacteriophage. Such differentiation is extremely useful for epidemiological purposes when the primary source of infection is the human carrier.

Phage typing of Vi-containing strains of $Sal.\ typhi$ is applied on an international scale, and $Sal.\ paratyphi\ A$ and $Sal.\ paratyphi\ B$ are also typed but to a lesser extent. The phage types of the typhoid bacillus are arbitrarily designated as type A, B, etc., with some subtypes, to type T, and there are nine additional types which are numbered or designated with a name, to give a total of 33 such types. Type E_1 is the most common all over the world, with type A a close second. In this country the commonest is type E_1 , followed in frequency by type C, type A, and type F_1 in that order, but about 10 per cent of strains cannot be typed.

The predominance of one or another of the phage types of Sal. typhi may characterize a geographical region. In the Western Hemisphere, types C, E₁, D₁, and A occur in that order in Canada, and types A, T, and E₁ in Venezuela. In England the most frequently occurring types are E₁, A, C, and D₁; in Denmark A, F₁, E₁, and C; in Portugal B₃, A, E₁, T, and D; etc. Other phage types are discontinuously distributed; viz., type M is found in Asia, in fact is commonest in Vietnam, and otherwise is found in Iran, France, and Poland.

There are four phage types of Sal. paratyphi A and 10 types of Sal. paratyphi B^{57, 62, 66} in both series designated by numbers. These have been less extensively studied than the phage types of Sal. typhi, and in both groups type 1 appears to be the commonest wherever studies have been

ECOLOGY 501

made. Other Salmonella serotypes have not been studied in any detail with respect to phage types, except *Sal. typhimurium*^{12, 33} of which some 80 phage types are distinguishable.

Ecology. 56, 60 As noted earlier, bacteria of the Salmonella group all appear to be obligate parasites, and they are not found apart from animal hosts. Sal. typhi, Sal. paratyphi A, and usually but not invariably Sal. paratyphi B are restricted to man. where they cause disease or persist in the carrier state. The remainder of this large group are parasites of lower animals, especially rodents and birds, although they are found occasionally in reptiles. The natural host of Sal. enteritidis, for example, seems to be the rat, and that of Sal. typhimurium, the so-called mouse typhoid bacillus, the mouse. Fowl are commonly infected. including chickens, turkeys, and ducks, and may constitute the largest reservoir of human infection; of other domestic animals, the pig is most commonly infected, especially with varieties of Sal. cholerae-suis, which is not, however, the etiological agent of hog cholera, a virus disease. Of a series of 2520 isolates²³ exclusive of Sal. pullorum and Sal. gallinarum, the causative agents of bacillary white diarrhea of chicks, 1258 were isolated from fowls, 532 from man, 475 from swine, 90 from rodents, 88 from carnivores, 53 from horses, and 20 from ruminants. These proportions reflect the kind of specimens examined and hardly constitute a random sample.

The isolates from human sources in this series include 41 types of Salmonella; of those occurring most frequently, 132 were Sal. paratyphi B, 60 were Sal. typhimurium, 53 were Sal. newport, 37 were Sal. newington, 28 were Sal. cholerae-suis var. Kunzendorf, 27 were Sal. panama, and 24 were Sal. montevideo. These proportions are probably reasonably representative, but one type or another may predominate in man in a given locality for a time and then, unaccountably, shift. For a period of several years, for example, Sal. montevideo was found in the great majority of specimens examined at the University of Chicago Clinics.

The presence of Salmonella is frequently associated with disease, both in man and in lower animals, and epidemics of Salmonella infection may occur in flocks of

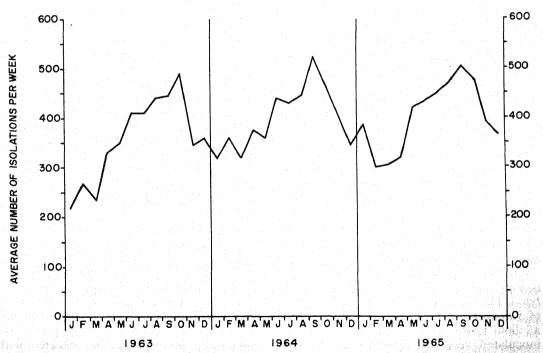


Figure 106. The incidence of Salmonella infection in man as indicated by the number of reported human isolations in the United States over the period 1963-1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

turkeys and similar situations. The infection may also be symptomless and the host act as a carrier, but the carrier state is usually not chronic except in the case of chronic human carriers of *Sal. typhi* where the gall-bladder or urinary bladder is infected, ap-

parently permanently.

Bacteriological diagnosis of Salmonella infection.25,42 The differentiation of the paratyphoid fevers from typhoid fever and the determination of the etiology of gastroenteritis caused by Salmonella is necessarily dependent on the isolation and identification of the causative microorganism. For isolation both enrichment culture and direct plating should be used; enrichment broth and differential selective agar plates should be inoculated simultaneously and, if the latter are negative, fresh plates can be inoculated from the enrichment culture. It is commonly observed that no single agar medium suffices, for with very few bacteria cultures may be isolated on one medium but not the others; at least two, and better three, kinds of differential agar should be used.

Two enrichment mediums are commonly used. Selenite-F broth contains 0.4 per cent sodium acid selenite, which is toxic for all gram-negative bacteria but is detoxified sufficiently in the presence of 1 per cent sodium phosphate to permit rapid growth of enteric pathogens while temporarily (eight to 12 hours) inhibiting E. coli.67 Tetrathionate broth contains thiosulfate, tetrathionate, and iodide, the tetrathionate being formed by oxidation of the thiosulfate with iodine. The differential selective agars used contain lactose and an indicator (often neutral red), together with bile or bile salts.38 Of these the most commonly used are desoxycholate-citrate agar (D-C), Shigella-Salmonella agar (S-S), and MacConkey agar. On these mediums the nonlactosefermenting bacteria form colorless opaque or translucent colonies which are readily differentiated from the red colonies of the lactose-fermenters.

The Wilson-Blair bismuth-sulfite medium is especially useful for the isolation of Sal. typhi. The colonies appear black, as do those of Sal. paratyphi B and Sal. enteritidis. The medium is strongly inhibitory and must be inoculated very heavily; consequently the colonies may not be pure cultures.

Typical colonies are picked and subcul-

tured in Kligler's iron agar or triple sugar iron agar; these mediums indicate acid or acid and gas formation from the sugars present and also the formation of hydrogen sulfide. After 24 hours' incubation, the growth is subcultured in urea medium, citrate medium, motility medium, gelatin, and lactose, sucrose, and mannitol broths. Lactose broth cultures should be retained for not less than two weeks to eliminate slow lactose fermentation. A positive lactose (and/or sucrose) fermentation, hydrolysis of urea, or indol formation eliminates Salmonella.

Further identification is serological.²² first with polyvalent antiserum which does not, however, eliminate certain of the dysentery bacilli (Flexner types 1 and 2) or serologically related paracolon bacilli of the Arizona group. Serological identification is made more precise by typing with monospecific serums as indicated earlier, either approximately or in detail. The usual technique is a rapid slide agglutination in which a drop of relatively heavy (milky) suspension of the bacteria is placed on a slide and mixed with a drop of antiserum, which may be appropriately diluted to eliminate crossreactions in some cases. Within a few minutes agglutination occurs, and the milky suspension appears to become curdled. This is most readily observed under low magnification, such as with a hand lens or a dissecting microscope. It is essential that a saline control be included, for rough forms agglutinate spontaneously in salt solution.

Pathogenicity for lower animals. Salmonella infection of rodents is quite common; Sal typhimurium and Sal. enteritidis cause infections of rats and mice, and these animals may become healthy carriers of the bacilli, a point of importance in connection with the epidemiology of food-poisoning outbreaks. Sal. typhimurium infection is by far the most common in the United States, and Sal. enteritidis less so than generally supposed. Preparations of "rat virus" or "Ratin" consist of these bacteria and are supposed to initiate an epidemic of disease in the rat population and hence destroy it. Not all the rats are killed and many of the survivors become healthy carriers.

Contrary to what had been previously supposed, the dog may be infected with Salmonellas; as high as 15 per cent incidence has been observed. While it seems unlikely

that the dog is an important reservoir of human infection, it has been found to transmit infection to man.¹³

Salmonella infection of the horse is also quite common. Infectious abortion of mares is caused by a specific microorganism, Sal. abortus equi, which has not been found in other animals. Man is only rarely infected. Sal. typhimurium has also occasionally been reported in horses. Abortion in sheep has been attributed to a member of the Salmonella group, Sal. abortus ovis. Salmonella is occasionally observed in a variety of other animals.

Birds are quite commonly infected with members of the Salmonella group. Epidemics due to Sal. typhimurium sometimes cause great destruction among canaries and other songbirds. Salmonella infections in turkeys occur and may be of such magnitude as to assume both economic and public health importance.1 Two barnyard diseases of great economic importance are due to specific Salmonella types: the bacillary white diarrhea of chicks caused by Sal. pullorum and fowl typhoid caused by Sal. gallinarum. Sal. pullorum may survive in the ovaries of fowls that recover from infection; diseased chicks may develop from the infected ova and communicate the disease to initially healthy members of the flock. Rare cases of human infection with Sal. pullorum have been reported, and it has been associated with epidemic foodborne gastroenteritis in some instances.

The typhoid bacillus. The typhoid bacillus is, in contrast to most members of the Salmonella group, almost completely non-pathogenic for lower animals except the chimpanzee, in which a disease closely

resembling typhoid fever is produced by oral inoculation. ¹⁹ There have been a number of accidental laboratory infections in man and, with the advent of chloramphenicol as an effective chemotherapeutic agent, the disease has been reproduced in human volunteers. The infecting dose for man in the latter kind of experiment has been found to be 10⁶ to 10⁷ bacilli.

The lethal dose for the mouse by the intraperitoneal route is of the order of many millions of bacilli and approaches the toxicity of the killed bacteria. Virulence for the mouse may be greatly increased by suspension of the bacilli in 5 per cent mucin, and the LD₅₀ by the intraperitoneal route may be on the order of 103 bacteria. The disease produced is a fulminating bacteremia, usually resulting in death within 48 hours. This kind of experimental infection has had wide application in assay of the immunogenic potency of vaccines and study of the relative importance to immunity of the Vi antigen and various fractions of the O antigenic complex.

A fatal infection with relatively small numbers of bacilli may be produced in the mouse by intracerebral inoculation.⁴⁷ The infection disseminates in the central nervous system to produce an acute purulent meningoencephalitis with secondary edema and pyrocephalus. Guinea pigs may be infected by direct inoculation of the gallbladder, and some animals so infected may become chronic carriers.⁴¹ This kind of infection, first described by Bingel with dysentery bacilli, has been used as a criterion of pathogenicity for a number of the Enterobacteriaceae.⁶⁹

Salmonella Infections

Salmonella infection in man is almost always acquired by ingestion of the microorganisms, usually as contamination of food, milk, or water. Occasionally double infections occur, *i.e.*, simultaneous infections with more than one kind of Salmonella, ⁶⁵ but these are not common. Two kinds of disease occur, one an acute gastroenteritis characterized by vomiting and diarrhea in which a small portion of patients become septicemic, and the bacteria are

found in the blood in relatively large numbers. The other is the typhoidal or continued fever type of disease in which bacteremia occurs in the initial stage of disease, and the symptoms are more general in nature. Sal. typhi, Sal. paratyphi A, usually Sal. paratyphi B, occasionally Sal. paratyphi C and related forms, and exceptionally almost any other Salmonella serotype, are responsible for the continued fevers. Acute gastroenteritis, on the other hand, is seldom if

ever due to infection with Sal. typhi or Sal. paratyphi A, and a minor portion of cases of infection with Sal. paratyphi B are of this kind, but the great majority of cases of gastroenteritis of Salmonella etiology are due to infection with the multiplicity of other Salmonella serotypes, largely Sal. typhimurium, Sal. enteritidis, Sal. cholerae-suis, etc. While, then, no sharp distinction can be made between the etiological agents of these two kinds of diseases, there is a general association.

In addition to these main types of disease, a wide variety of diseases may occasionally be associated with Salmonella infection.9 These microorganisms all have a predilection for the bone marrow, and bone infections are not uncommon, together with other localized infections, infections of the central nervous system, etc. A carrier state, usually transitory except in the case of chronic carriers of Sal. typhi (see below), occurs in man with discharge of the microorganisms in the feces. Infection may be acquired from human carriers, and such carriers should not, for example, be food handlers, but probably most human infections are from lower animals, viz., contamination of food with rodent feces, contamination of bulk and dried eggs by infected fowl, etc.

SALMONELLA GASTROENTERITIS

Gastroenteritis of Salmonella etiology is characterized by a short incubation period, as little as 12 hours in some cases, acute vomiting and diarrhea with slight rise in temperature, and rapid recovery, usually within a few days. Occasionally this kind of disease develops into a more serious septicemic condition, and there is a greater tendency to do so when the infectious agent is Sal. cholerae-suis and related forms of the "para C group." It is most often acquired by ingestion of contaminated food, and is called Salmonella food poisoning, but is an infection rather than an intoxication as in staphylococcal food poisoning. Salmonella food poisoning seems to be relatively more common than staphylococcal food poisoning in England, and the reverse in this country, but it is not clear that there is a real difference. 16, 17 No doubt some, and possibly many, of the descriptions of indigenous

cholera and cholera nostra in earlier writings refer to this kind of disease.

The general behavior of Salmonella infection is illustrated in a study of 7779 strains of Salmonella isolated between 1937 and 1955.61 In this group, gastroenteritis occurred in 68 per cent of the cases and was appreciably more severe in infants and persons over 50, and a typhoidal or septicemic condition occurred in 8.8 per cent. The over-all case fatality rate was 4.1 per cent, with more than half the fatalities occurring in infections with Sal typhimurium and Sal. cholerae-suis; in the latter infection, which tended to be septicemic, the case fatality rate was 20.3 per cent. In Sal. enteritidis infections, the case fatality rate was 5.8 per cent for all ages, but 15 per cent in those over 50.

Salmonella typhimurium (Salmonella aertrycke, Bacterium aertrycke, Bacterium typhimurium). This bacterium, most commonly isolated in food poisoning outbreaks in the United States and in Great Britain, closely resembles Sal. paratyphi B in its cultural characteristics but can be distinguished by its ability to produce acid in tartrate medium. Before differential tests were satisfactorily worked out, Sal. paratyphi B and Sal. typhimurium were commonly confused and both termed "para B." Sal. typhimurium is commonly found in a variety of infections in laboratory and domestic animals and in birds, and the "B. pestis caviae" of some writers and Nocard's "B. psittacosis" are, in fact, Sal. typhimurium. Most of the laboratory stock cultures labeled "Danysz virus" or "bacillus of mouse typhoid" are of the typhimurium type, but some are Sal. enteritidis.

Salmonella enteritidis (Bacterium enteritidis). Although found frequently in food-poisoning outbreaks, this bacterium is less common than Sal. typhimurium. It closely resembles Sal. typhimurium culturally but is said by some to differ in that it does not ferment inositol while typhimurium does. The inositol fermentation is, however, frequently not clear-cut; a lowering of pH may be noted but sometimes not to a sufficient degree to warrant calling the fermentation positive.

Salmonella cholerae-suis (Bacterium suipestifer, Bacterium cholerae-suis, the hog cholera bacillus, American suipestifer). This Salmonella is a member of a group of closely related bacteria, called the suipestifer group, which also contains Sal. paratyphi C or Eastern type, as noted above: Sal. cholerae-suis var. Kunzendorf or European type; and the Glässer-Voldagsen type, which is comprised of two species, Sal. typhi-suis and Sal. typhi-suis var. Voldagsen. These species may be differentiated from one another by a combination of cultural and serological methods. Sal. cholerae-suis formerly predominated in the United States. but now the Kunzendorf variety is found more often. This species has been implicated in outbreaks of paratyphoid gastroenteritis. although to a much lesser extent than either Sal. typhimurium or Sal. enteritidis. Sal. typhi-suis appears to be an animal (pig) pathogen, but several cases of human infection with the Voldagsen variety have occurred in the United States. The case fatality rate in cholerae-suis infections is 20 to 25 per cent in contrast to 5 per cent or less in infections with other Salmonellas

Other Salmonella species are less commonly implicated in human infections. Sal. thompson and Sal. newport, both related to the suipestifer group immunologically, have been observed a few times and other species but once; in fact, a number of the new Salmonella species described in recent years have been isolated from food implicated in outbreaks of enteritis.

TYPHOID AND PARATYPHOID FEVERS

Salmonella infection resulting in the continued fever kind of disease is characteristically typhoid fever, caused by Sal. typhi. As indicated above, the infectious nature of typhoid fever was suspected by the middle of the last century, and the causative agent was among the earlier of the pathogenic bacteria to be described. Within a few years paratyphoid fever was differentiated. Achard and Bensaude isolated a microorganism similar to, but not identical with, the typhoid bacillus from typhoid-like disease in 1896, and two years later Gwyn isolated a microorganism closely resembling Sal. enteritidis under similar circumstances. Since that time a number of investigators in different parts of the world have isolated similar bacteria from the blood of patients suffering from a disease that, so far as clinical symptoms are concerned, is substantially identical with typhoid fever.

Paratyphoid fever. Many cases of paratyphoid infection show a tendency to run a relatively mild course and are marked by a sudden onset with chills, but are otherwise very similar to infections with the typhoid bacillus. A certain proportion of the negative agglutination tests in apparent typhoid fever are probably a result of paratyphoid infection. The only method by which typhoid and paratyphoid fever can be distinguished is isolation and identification of the causal microorganism.

Many scattered cases of paratyphoid fever have been observed, and a number of more or less extensive epidemics have been reported as due to milk and other foods, to contact with human carriers, to sewage-polluted water and similar factors. Infection is probably most often acquired from carriers; the bacilli may be excreted for some weeks by convalescents, and subclinical undiagnosed cases are similarly a source of infection.³⁷ In general, the mode of dissemination of paratyphoid fever is practically identical with that of typhoid fever.

The frequency of the paratyphoid fevers as compared with typhoid fever varies a good deal in different localities, but most hospital records give a ratio of less than 1:10. In some regions the proportion of paratyphoid to typhoid may be as high as 1:4 or even more. During the First World War the proportion of paratyphoid to typhoid reached a high point. In the British armies in France during the years 1915 to 1918, the diagnosed paratyphoid fevers outnumbered the typhoid cases 2:1. In civilian populations in most countries paratyphoid fevers probably amount to 5 or 10 up to 50 per cent or more of all fully diagnosed enteric cases. Very young individuals appear to be the most susceptible to Salmonella infection, and an unduly large portion, perhaps 20 per cent, of cultures are from young children.

Salmonella paratyphi A (Bacillus paratyphosus A, Bacterium paratyphosum A, Salmonella paratyphi). This bacterium differs culturally from most of the other Salmonella species in its inability to ferment xylose, and it is, in addition, serologically distinct. Some outbreaks due to this microorganism have been traced to sewage-contaminated water supplies, others to food contaminated through the agency of human carriers. Paratyphoid fever due to Sal. paratyphi A is often very mild, 300 cases occur-

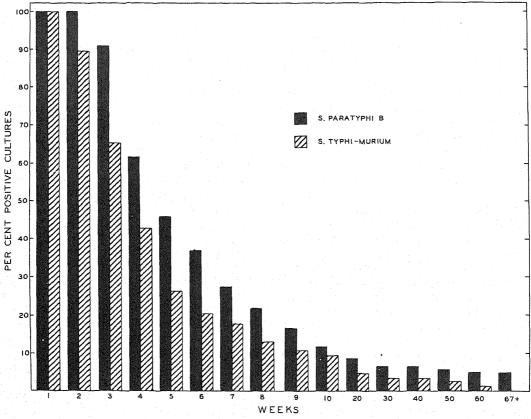


Figure 107. The persistence of Salmonella infection as indicated by positive fecal cultures by weeks after onset. (Data on 203 cases of Salmonella paratyphi B infection and 239 cases of Salmonella typhimurium infection, by Rubenstein, Feemster, and Smith. 59)

ring in a United States infantry regiment without a single death.

Salmonella paratyphi B (Bacillus paratyphosus B, Bacterium paratyphosum B, Salmonella schottmülleri). Sal. paratyphi B is readily differentiated from Sal. paratyphi A, but has confusing cultural and serological relations with certain Salmonella strains of the food poisoning types. As in the preceding species, the sources and modes of transmission are similar to those of typhoid fever. The source of the bacteria is generally the human carrier, but it has been reported that dogs have been found responsible for small epidemics, and in another instance a cow was responsible for cases of the disease. In the northern United States and in northern Europe infection with this species seems considerably more frequent than with Sal. paratyphi A.

Salmonella paratyphi C (Sal. hirschfeldii). Sal. paratyphi C has been found in enteric fevers in parts of Asia, Africa, and southeastern Europe and has been reported as an important cause of illness and death in British Guiana. Cases of endocarditis have been found to be caused by this and other suipestifer species. Although the type of disease is similar to the other enteric or intestinal forms, little is known about its epidemiology. This species is closely related biologically to the hog cholera or cholerae-suis strains described above.

Other species have been occasionally found: Sal. barielly, isolated from cases of mild pyrexia in India; Sal. enteritidis var. moscow, isolated from cases of paratyphoid fever in Russia and described by Russian bacteriologists as "Paratyphus N2"; Sal. sendai, isolated from cases of paratyphoid fever in Japan; and Sal. eastbourne, from a paratyphoid case in Eastbourne, England. In this country Sal. typhimurium, Sal. saint paul, Sal. oranienburg, Sal. hartford, Sal. sendai, Sal. panama, and others have been found associated with enteric fevers.

Typhoid fever.⁴³ Typhoid fever was for long one of the most widespread and im-

portant of all bacterial diseases. In the United States in 1900 there were 35,379 reported deaths from this disease, undoubtedly a low figure, and probably some 350,000 cases of typhoid fever in a population of 76.000.000 — in the course of a decade perhaps one person in every 20 to 25 contracted the disease. The prevalence of typhoid fever has greatly diminished in recent years, and a large part of this decrease has taken place in the large cities. Typhoid still persists, however, and epidemics occur from time to time, particularly in the smaller towns and rural areas. Much the same situation prevails in other countries, such as Great Britain.

The common symptoms of typhoid fever include frontal headache, lack of appetite, nosebleed, the development of rose spots on the abdomen, muscular weakness, and diarrhea. Sometimes considered primarily an intestinal infection, the disease is, in fact, a general invasion of the body, particularly of the lymphatic system. The gastrointestinal tract is the portal of entry of the bacilli into the body; the lymphatic tissues in the intestinal wall are first invaded. and the bacilli spread through the lymphatic system. After considerable multiplication has occurred (incubation period), the bacilli overflow into the blood and bacteriolysis takes place; the endotoxins which are liberated as a result of the destruction of the

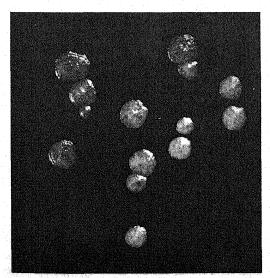


Figure 108. Colonies of typhoid bacillus on nutrient agar. Note the characteristic "maple leaf" irregular margin and slightly roughened glistening surface. × 6.

bacilli produce the symptoms of typhoid fever. Other evidence suggests that the body tissues may be invaded through the tonsils and gastric mucosa, but in any case the end result is the same, and typhoid fever is a general and not a localized infection.

The typhoid bacillus appears in the blood stream early in the disease, i.e., after the onset of symptoms, and may be cultured within the first 10 days in the majority of cases, either from a blood sample or from the clot of samples sent into a laboratory for agglutination tests.80,81 The presence of typhoid bacilli in the blood, however, does not constitute septicemia; in fact, probably little or no multiplication takes place. The bacilli are also present in the bone marrow early in the disease, and some have urged culture by sternal puncture to facilitate diagnosis. During and after the second week typhoid bacilli may be found with increasing frequency in the feces, and the proportion of positive blood cultures drops off. The bacilli are also excreted in the urine in perhaps 25 per cent of the cases. They may often be found early in the disease in the rose spots, not in the blood but in the lymphatic spaces.

On autopsy, the intestinal walls are usually found to be extensively ulcerated, Peyer's patches and the solitary glands of the intestine being particularly involved and containing typhoid bacilli. Perforation of the intestinal wall as a consequence of ulceration is a not uncommon occurrence. The spleen is enlarged and congested and usually contains large numbers of typhoid bacilli. In both the spleen and the liver the bacilli seen in stained sections occur in groups or masses rather sharply focalized; scattered individuals are not often found.

A variety of complications may occur. Laryngeal ulcer is occasionally observed. The gallbladder is often infected, and cystitis sometimes occurs. Suppurative and inflammatory processes may appear in other parts of the body. The osseous system seems especially open to attack, and affections of the periosteum, the bone marrow, and the joints have been traced to infection with Sal. typhi. Osteomyelitis may develop as long as six or seven years after recovery from typhoid fever, indicating that the typhoid bacillus can remain in contact with human tissues for years without losing its virulence. Other parts of the body are more rarely invaded during typhoid fever, but almost any

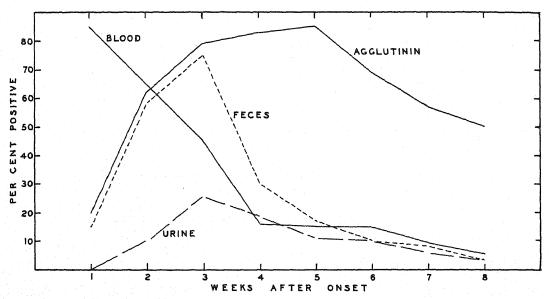


Figure 109. The approximate incidence of positive culture of blood, feces, and urine and the agglutinin response in typhoid fever.

organ can be attacked occasionally. The presence of *Sal. typhi* has been reported in a brain abscess, and the cerebral and meningeal symptoms occurring in many cases of typhoid fever are directly connected with the localization of the bacilli in the meninges; bacilli have been found in spinal fluid obtained by lumbar puncture. Pulmonary involvement may occur also, possibly more often than has been supposed.⁵⁵

Secondary or mixed infections, especially with the pyogenic cocci and the pneumococcus, are not at all uncommon and sometimes result in serious complications. Mixed infections with the tubercle bacillus and the anthrax bacillus have also been observed.

Chemotherapy. The typhoid bacillus is relatively resistant to penicillin in vitro, but although it is susceptible under these conditions to the other common antibiotics, chloramphenicol appears to be the only one that is reasonably effective in vivo. It is, therefore, the chemotherapeutic agent of choice and has given highly promising results. It has been reported that relapses tend to occur unless the drug is given for two to four weeks. Some attempts have been made to cure the carrier state by chemotherapy, with encouraging results in some instances but-not in others.⁷⁸

Carriers.⁵¹ About one-third of the individuals having typhoid fever discharge bacilli for a period of three weeks after the onset of illness and about 10 per cent for

eight to 10 weeks; these are known as convalescent carriers. A certain proportion continue to discharge typhoid bacilli for six months or more, and in many cases over a period of several years or throughout the whole of a long life.

The development of the carrier condition is probably dependent upon the invasion of the gallbladder in the case of the fecal carriers and of the urinary bladder in the case of the urinary carriers. Fecal carriers are more common than urinary carriers, and combined fecal and urinary carriers are relatively uncommon. The development of the urinary carrier state is apparently favored by pathology of the urinary tract produced, for example, by urinary schistosomiasis, and this probably accounts at least in part for the high incidence of urinary carriers in certain areas such as Egypt.39 Urinary carriers have been found to excrete antibody in the urine.4 It is not known why women are more commonly carriers than men. In the series studied by Ames and Robins,² 2.1 per cent of the males became chronic carriers as compared with 3.8 per cent of the females. Age is a factor also; according to the same workers the percentage of cases becoming carriers was 0.3 in the 0 to 9 and 10 to 19 age groups, but as high as 10.1 in the 50 to 59 age group. The usual estimates for all age groups vary from 0.5 to 11.6 per cent.

Typhoid bacilli need not be excreted con-

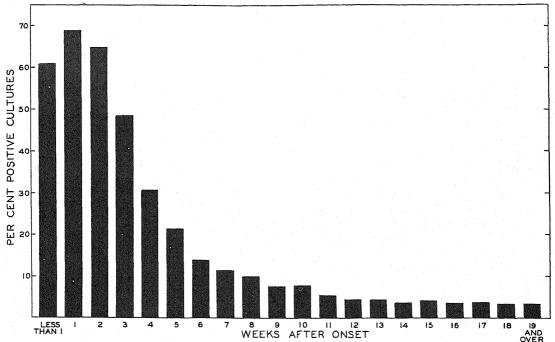


Figure 110. The persistence of typhoid bacillus infection as indicated by percentage of positive fecal cultures by weeks after onset. (Data from 374 cases in New York State, exclusive of New York City, by Ames and Robins.²)

tinuously; in fact, their intermittent appearance is very common, and weeks may elapse with negative cultures before the bacilli reappear. 10 The necessity for repeated examinations is obvious. The numbers of bacilli may vary widely, from 500,000 to 450,000,000 per gram of feces in one study.⁷⁴ A majority of carriers give the Widal reaction, and in most cases the opsonic index is abnormally high. Antibody to Vi antigen is found in the great majority of carriers but is only transitory if present at all in inoculated persons, and the use of the Vi agglutination test for the detection of carriers has given encouraging results64 but is not accepted as established.

Attempts to cure typhoid carriers by nonsurgical means, such as chemotherapy, vaccine therapy, or bacteriophage, have not been generally successful, and such procedures are not generally advocated at the present time. Removal of the gallbladder under suitable conditions is often effective in the case of fecal carriers; 70 per cent is the usually accepted figure. The chemotherapy of the carrier state is noted above.

The proportion of typhoid carriers in the general population is not known, owing to obvious practical and technical difficulties. It is probably quite different in different

localities and doubtless depends largely upon the prevalence of current and past typhoid infection. By a modified life table procedure Ames and Robins² estimated a carrier rate of 42 per 100,000 in New York State. On the basis of reported deaths and assuming a case fatality rate of 10 per cent and a chronic carrier incidence of 2 per cent in survivors, carrier rates have been estimated as 48 per 100,000 in Massachusetts and 288 per 100,000 in Mississippi.

The number of carriers throughout the United States is probably decreasing since they are no longer produced in such quantities as when typhoid fever was prevalent. By extrapolation Ames and Robins² calculated that the 2500 carriers in New York State in 1940 would be reduced to 200 by 1980.

Epidemiology. The typhoid bacillus is a strict parasite found only in man. Outside the human body multiplication, if it occurs at all, is insignificant, and for practical purposes may be neglected as a factor in the dissemination of the disease. As indicated above, the typhoid bacillus leaves the body in the feces or, less commonly, the urine, and enters the body of a new host via the alimentary tract. The epidemiology of typhoid fever, then, is predicated upon the

connection between the intestinal tract of the infected person and the mouth of the susceptible, and the factors that determine the spread of this disease are, essentially, those arising as a consequence of the interrelationships of the individuals or groups of individuals comprising the host population. The extent of the spread of typhoid is dependent upon the nature of the connecting links between individuals, and two epidemiological types of the disease may be distinguished, the one epidemic typhoid, the other endemic, or residual, typhoid.

Epidemic typhoid fever. Extensive outbreaks of typhoid fever necessarily involve a connecting link that is common to a great many people, and by far the most important vectors of this kind are water and milk. As pointed out elsewhere (Chap. Ten), waterborne typhoid fever, formerly all too common but by now relatively rare in the larger communities, occurs as a consequence of the contamination of a water supply with infectious fecal material, either as such or in the form of sewage. Waterborne epidemics of typhoid fever occur in the absence of chlorination, filtration, and other purification procedures and may be readily prevented. These epidemics tend to occur in the cold months of the year, particularly in the winter and early spring, and the incidence of the disease is unaffected by age, sex, or economic status. Such epidemics still occur occasionally, as in Zermatt in 1963.8

Milkborne typhoid fever, at the beginning of the twentieth century second only to waterborne typhoid in extent and importance, follows the route of the milkman and, as might be expected, tends to occur in the lower age groups and in families of higher economic status. The general use of pasteurization has practically eliminated milkborne typhoid fever.

Foodborne typhoid fever may take on epidemic proportions in certain instances. An epidemic due to infected corned beef, for example, occurred in Aberdeen, Scotland in 1964.⁷⁹ Oysters and other shellfish have come into bad repute in this respect, for a number of typhoid epidemics in Great Britain and the United States have been found to be due to the eating of oysters grown near sewer outfalls or placed to "fatten" in the polluted waters of estuaries or creeks. Water cress, lettuce, radishes, or any vegetables or fruits which are liable

to come in contact with contaminated water or are sprayed with human excrement may give rise to small-scale epidemic typhoid fever.

Endemic typhoid fever. Although epidemic typhoid fever is largely eliminated in a given community through adequate sanitary control of water and milk supplies and such food supplies as are susceptible to the application of effective control measures, the disease remains in an endemic form which is manifested as occasional cases or small groups of cases which appear from time to time. The seasonal incidence is quite different from that of waterborne typhoid; for some years there has been a sharp peak in the summer, but with continued reduction in the incidence of the disease, this has tended to disappear to give a relatively even distribution of cases throughout the year. The source of infection is, of course, the case, frank or ambulatory, or the healthy carrier. Instances of direct contact infection are unquestionably more common than is generally recognized, and the dissemination of typhoid bacilli from the infected individual to his immediate associates is undoubtedly responsible for the majority of cases of residual typhoid. Carriers are of particular importance in that they constitute semipermanent foci of infection and, when employed as food handlers, may be the cause of small epidemics. The most notorious instance of this kind was that of Mary Mallon, "Typhoid Mary," who was unknowingly the cause of some 26 cases of typhoid fever in seven different families. Under special circumstances, such as prevail among troops, contact infection may assume epidemic proportions.

The reduction in the prevalence of typhoid fever in the last few decades is attributable almost entirely to elimination of the great waterborne and milkborne epidemics. As noted above, epidemics still occur, and their control is a matter of putting into practice existing knowledge. Residual typhoid, however, is much more difficult to control; the detection and supervision of all carriers, or even the elimination of carriers as food handlers, is a practical impossibility. There is reason to believe that with continued control of epidemic typhoid fever. the reduction in the proportion of carriers may well be reflected in a reduced incidence of the disease in the epidemic form.

Immunity. An attack of typhoid fever

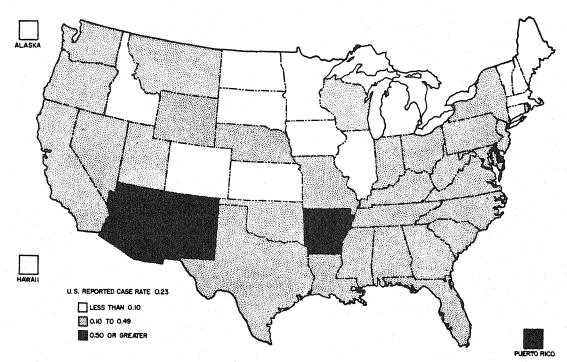


Figure 111. Geographical distribution of typhoid fever as cases per 100,000 in the United States in 1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

confers a certain degree of immunity, although instances of two or even more attacks in the same individual are not unknown.35 Animal experiment has shown that it is possible to obtain a high degree of immunity in rabbits and guinea pigs against intraperitoneal inoculation. Recovery from experimental enteric infection in the chimpanzee is also associated with an immunity to subsequent challenge by the oral route, 18 months after the primary infection.36 The immunity is associated with the development of humoral antibodies such as agglutinins, precipitins, and the like. Lysin is also produced, and, like the cholera vibrio, the typhoid bacillus undergoes visible dissolution and disintegration in the peritoneal cavity of the immune animal.

In man recovery from typhoid fever is also accompanied by the appearance of demonstrable antibodies. In most instances agglutinins appear during the course of the disease, sometimes as early as the fifth day (over 90 per cent by the fourth week), and their presence is the basis of the Widal test used for diagnostic purposes.

The Widal test.⁴⁴ In its original form the Widal test was a slide agglutination test, and agglutination of typhoid bacilli by pa-

tient's serum in a dilution of 1:50 or more was considered positive. The development of knowledge of the antigenic structure of the typhoid and paratyphoid bacilli in recent years has resulted in a somewhat better understanding of the value and limitations of the agglutination test in the diagnosis of typhoid fever. At the present time the test is a macroscopic one and is carried out with both H and O antigens. The interpretation of a single such test must take into consideration ancillary data such as a previous immunization or attack of typhoid fever and the prevalence of endemic typhoid in the general population. It is, therefore, difficult to set arbitrary limits; in most instances an O titer of 1:100 and an H titer of 1:200 may be regarded as significant. A point of some interest is the lack of sharp antigenic specificity of human serums as compared with the specificity of experimentally produced rabbit antiserums.

The interpretation of the Widal test in immunized persons is often difficult, since both H and O agglutinins are formed in response to the vaccine. Titers fall after immunization but may persist at moderate levels for many months. Furthermore, the agglutinin titer may rise in anamnestic response to a febrile

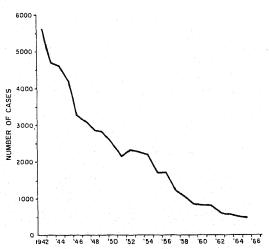


Figure 112. Cases of typhoid fever reported in the United States, 1942-1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

condition. The extent to which this occurs is not definitely known, and some data suggest that it is significant while others do not. Such an anamnestic reaction is particularly prone to occur in typhus fever, and typhoid agglutinin titers as high as 1:800 have been observed. There is some evidence that an "agglutinin curve," obtained by periodic agglutinin titrations, has diagnostic value in that it continues to rise in typhoid fever but usually does not do so in the anamnestic reaction.

Prophylactic inoculation. 6, 30 vaccine has been used for many years to produce an active prophylactic immunity. The efficacy of such immunization is indicated by the marked reduction in incidence of the disease by mass inoculation, which is particularly applicable in military services. At the time of the Spanish-American War in 1898, 4422 cases of typhoid fever and 248 deaths occurred in a division of 10,759 men. Typhoid immunization has been compulsory in the United States Army since 1911, and has resulted in the practical disappearance of the disease. 11 The immunity so produced may be broken down, and typhoid fever in immunized military personnel has been observed from time to time^{3, 71} but is rare.

The vaccine consists of a saline suspension of typhoid bacilli, usually 1000 million per milliliter, which are killed by heat, phenol, formaldehyde, etc. Triple vaccine con-

taining para A and para B bacilli in addition to typhoid bacilli (TAB vaccine) to the same total concentration but in a ratio of 2:1:1 has been commonly used, but the desirability of including the paratyphoid bacilli at the expense of half the typhoid antigen is questioned from time to time. For primary immunization, the vaccine is given as three doses, 0.5, 1.0, and 1.0 ml., at weekly intervals. A single inoculation, 1.0 ml. subcutaneously or 0.1 to 0.2 ml. intradermally, within two to three years, suffices for booster inoculation.

Questions of the assay of the immunogenic potency of typhoid vaccines, and their antigenic composition are raised perennially. There are discrepancies between the results of field trials, studies of the experimental disease, and immunogenic potency tests, and no final answers are as yet possible.

The most commonly used immunogenic potency test is the mouse protection test, either active or passive. In this country groups of 30 mice are immunized with 0.5 ml. of a 1:10 dilution of the vaccine intraperitoneally, and challenged, in groups of 10, two weeks later with 1000, 10,000, and $100,000 \text{ LD}_{50}$ in the mucin-potentiated infection. At least 50 per cent of the mice should be protected against not less than 10,000 LD_{50} . Intracerebral challenge has been studied but is not generally used. Passive protection tests, carried out in a similar way, may be used to assay protective antibody in human or other serums.

Virulence of typhoid bacilli for the chimpanzee is associated with Vi antigen, ¹⁹ non-Vi strains producing only very mild disease, ⁷⁶ and the results of mouse protection tests have also indicated the greater immunogenic potency of Vi-containing typhoid vaccine. On the basis of the mouse test ⁷⁵ and the chimpanzee infection, ⁷⁷ it is apparent that antibody to the O-Vi antigenic complex is protective, while antibody to the H antigens is not.

A series of field trials have been carried out in British Guiana, Poland, Yugoslavia, and the USSR under the auspices of WHO, in which heat-killed vaccines preserved with phenol, alcohol-treated vaccines, and acetone-killed vaccines were used. The USSR vaccines also included an adjuvant lipopoly-saccharide antigen. In general, the heat-killed vaccines, containing H-O antigen were superior to those containing only O antigen, an observation at variance with the

results of their mouse protection immunogenic potency assays.⁶⁸ The status of the mouse test thus becomes quite uncertain as a measure of immunogenic potency in man.

Passive immunization. The use of antityphoid serum for therapeutic purposes has been considered by a number of workers, but there is still no conclusive evidence as to its value. Whether or not "anti-endotoxic" serums or other typhoid antiserums confer a passive immunity to typhoid infection in man is likewise not established.

ALCALIGENES FAECALIS

Alcaligenes faecalis or Bacterium faecalis alcaligenes closely resembles the typhoid bacillus morphologically, culturally and even in its growth on Endo, Conradi-Drigalski, and malachite green differential mediums. It has been found in feces and in water. It differs from the typhoid bacillus in the possession of one or more polar, instead of many peritrichous, flagella, and in distinct alkali production in mannitol and litmus milk, and it fails to produce acid from dextrose and other carbohydrates. It has been suggested that *Bact. alcaligenes* is a form of Bact. fluorescens non-liquefaciens which has lost the function of pigmentation, and it does have affinities with certain of the plant pathogens and soil bacteria. Its systematic position has been examined critically by Conn who

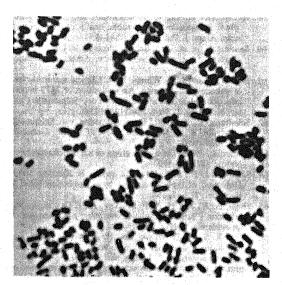


Figure 113. Alcaligenes faecalis. Smear from a pure culture. Fuchsin; × 2100.

has proposed a new genus, Agrobacterium, to include this bacillus together with Bact. radicicola and the bacterium of hairy root disease. Other species have been described as occurring in the intestinal tract, viz., A. metalcaligenes, A. bookeri, and A. recti, but these are not ordinarily differentiated from A. faecalis. Other varieties, A. viscosus and A. marshallii, are found in dairy products and produce a slimy alkalinity and ropiness in milk.

While A. faecalis is only feebly pathogenic, it has been found in cases of bacteremia, meningitis, infection of the gallbladder, renal tract, eye, lymph nodes, and appendix on occasion. Et has also been found to be the etiological agent of a disease closely resembling "red leg" (of Proteus etiology) in tree frogs. 53

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Chapter Twenty-two

THE ENTERIC BACILLI

The Dysentery Bacilli

Dysentery is a clinical rather than an etiological entity, and its characteristic symptoms, diarrhea, abdominal pain, and blood in the stools, may occur either alone or as part of the syndrome of a number of diseases. In the former instance dysentery may be of protozoan or bacterial etiology. In addition there is evidence suggesting that a filterable agent, presumably a virus, can cause dysentery in man. A dysentery-like infection may be produced by some members of the Salmonella group, but usually another group of bacteria, the dysentery bacilli, is responsible.³

The dysentery bacilli are gram-negative, nonspore-forming rods related to the other enteric bacteria. Some of them resemble the anaerogenic type of coliform bacilli and the typhoid bacillus in that they ferment carbohydrates with the production of acid and no gas. Others are related to the other coliform bacilli and paracolon bacilli. None of the dysentery bacilli are motile, and hence they do not contain the two types of antigens found in the paratyphoid bacilli. As a group they differ from one another biochemically and immunologically. In general, their uncertain relationship to the other enteric bacilli, coupled with their own heterogeneity, has made their classification a difficult matter.

The dysentery bacilli are facultative anaerobes, and their optimum temperature for growth is 37° C. Their nutritive requirements are not complex in that they will grow upon the ordinary nutrient (beef extract) mediums. In synthetic solutions nicotinic acid is apparently required by some strains. They ferment glucose to much the same end products as the other enteric

forms—lactic acid together with smaller amounts of formic and acetic acids and ethyl alcohol. Like the other gram-negative bacilli, they are relatively resistant to the bacteriostatic action of dyes, and these substances may be incorporated in differential mediums for their isolation; eosin—methylene blue (EMB) agar is commonly used.

Classification. The dysentery are divided into two groups on the basis of the fermentation of mannitol, the nonmannitol fermenters including the Shiga bacillus. This distinction was of early importance and continues to be so in tropical regions and elsewhere where the Shiga bacillus occurs, for the dysentery produced by it is much more severe and has a higher case fatality rate than the other bacillary dysenteries, but is not of practical significance in this country, where there is little or no Shiga dysentery. In addition to the Shiga bacillus, this group also includes the so-called parashiga bacilli and the Schmitz bacillus. The group of mannitol fermenters is further subdivided on the basis of slow (four to seven days) fermentation of lactose and the fermentation of dulcitol and sorbitol.

In the past the nomenclature of the dysentery bacilli has been somewhat casual, and informal names such as the Shiga bacillus, the Flexner bacillus, the Strong bacillus, and the Hiss-Russell Y bacillus have had wide currency. The nomenclature has been further complicated, especially within the Flexner group, by various individual designations and by the vacillations of various formal systems of classification. The name Shigella for the genus has gained reasonably wide acceptance, and the dys-

entery bacilli are regarded as species of this single genus. Finally international agreement has been reached, based on species names and serotypes as suggested by Ewing,¹⁷ to introduce a welcome order and stability.

Four groups of dysentery bacilli, designated A, B, C, and D, each comprising a single species name with serotypes where appropriate, make up the genus Shigella. These are:

(1) The nonmannitol-fermenting bacilli:

Group A. Shigella dysenteriae. Including the Shiga dysentery bacillus, the Schmitz bacillus, and the Large-Sachs group of parashiga bacilli as immunologically independent serotypes.

(2) The mannitol-fermenting bacilli:

- (a) Group B. Shigella flexneri. The Flexner dysentery bacilli, having characteristic cultural reactions and antigens in common, i.e., group antigens, but differentiable into numbered serotypes.
- (b) Group C. Shigella boydii. Culturally similar to the Flexner bacilli, but serologically unrelated to them or to one another, differentiable as independent numbered serotypes and including the Newcastle-Manchester bacilli.
- (c) Group D. Shigella sonnei. The slow lactosefermenting dysentery bacilli, or Sonne-Duval bacilli, and including the bacilli formerly known as Sh. ceylonensis A.

These groups do not include various other bacilli found in association with diarrheal disease and described as dysentery bacilli, such as Sh. alkalescens and Sh. dispar, which are considered to be coliforms, or bacilli such as Sh. arabinotarda types A and B, which are identical with group A serotypes.

The nomenclature used by Russian workers differs from the foregoing, a matter of some practical importance, but Ewing and his colleagues¹⁶ have brought the Russian nomenclature into juxtaposition with

the International Schema.

SHIGELLA DYSENTERIAE (GROUP A)

The dysentery bacilli making up this group are set apart by their inability to ferment mannitol. Sh. dysenteriae is immunologically heterogeneous, made up of 10 sharply separable serotypes arbitarily designated by numbers. The serotypes appear to be unrelated antigenically except for unilateral cross-reactions between some

strains of types 2 and 6, and some strains of types 3 and 5. Two of the serotypes, 2 and 10, are serologically related to *Sh. boydii* type 1 (see below) through the sharing of two or more factors, and type 8 is divisible into subtypes, 8a and 8b.¹⁵

Shigella dysenteriae type 1 (Bacterium dysenteriae, Shigella dysenteriae, Shigella shigae). This was the first of the dysentery bacilli to be described. It was found by the Japanese bacteriologist Shiga to be the etiological agent of epidemic dysentery in Japan in 1898, and was found by Kruse in Germany two years later. It subsequently became known that this bacillus had been isolated 10 years earlier by Chantemesse and Widal, who found it in postmortem cultures of bowel contents and mesenteric lymph nodes, but it had become established as the Shiga bacillus or the Shiga-Kruse bacillus.

The Shiga bacillus differs from the other dysentery bacilli in its marked toxicity for man and experimental animals. Two kinds of toxin are formed: an endotoxin closely bound to the cell substance which is a polysaccharide-lipid-polypeptide complex⁵³ that appears to have considerable immunizing activity as assayed by mouse protection, and an exotoxin found in filtrates of broth cultures, protein in nature and thermolabile. The exotoxin (neurotoxin) has an effect upon the nervous system and produces paralysis,46 while the endotoxin appears to act chiefly upon the alimentary tract. The neurotoxin is a true exotoxin12 and resembles diphtheria toxin in a number of respects. It is produced most abundantly in aerated culture, and its formation is affected by the iron concentration of the medium; toxin formation is prevented by 0.01 M iron and appreciably inhibited by as little as 0.000001 M. It has been prepared in highly purified form by van Heyningen and Gladstone³⁹ and found to be similar to diphtheria toxin in potency and in possible mode of action. The relationship of this toxin to the pathogenesis of bacillary dysentery of Sh. shigae etiology is not at all clear. The exotoxin, but not the endotoxin, may be inactivated by formaldehyde and the toxoid used as an immunizing agent. It has not yet been possible to destroy the toxicity of this or other dysentery bacillus endotoxins and at the same time retain antigenicity.

Shiga bacillus infections have been observed most frequently in India, Japan,

Shigella Group A Shigella dysenteriae

INTER- NATIONAL	OLDER	RUSSIAN NOMENCLATURE*				
TYPE	NOMENCLATURE	NAME	SEROTYPE			
I	Sh. shigae,	Grigorjeff-Shiga				
	Shiga-Kruse bacillus					
2	Schmitz bacillus,	Stutzer-Schmitz				
	Sh. ambigua					
3	Q 771	Bact. dysenteriae (Novgorodskaja-Semenova)	Roman			
4	Q 1167 Large-Sachs	Bact. dysenteriae (Novgorodskaja-Semenova)	1618			
5	Q 1030 group	Bact. dysenteriae (Novgorodskaja-Semenova)	819			
6	Q 454	Bact, dysenteriae (Novgorodskaja-Semenova)	Tjacht			
7	Q 902	Bact, dysenteriae (Novgorodskaja-Semenova)	2435			
8						
9	58					
10	2050-52					

^{*}According to Ewing and Trabulsi.19

China, and other parts of Asia; they appear to be relatively rare in the United States.

Shigella dysenteriae type 2 (Schmitz bacillus, Bacterium schmitzii, Shigella schmitzi, Shigella ambigua). This serotype was described by Schmitz in 1917 as a cause of dysentery in a Rumanian war prison camp. It does not ferment mannitol but differs in that it produces indol and ferments sorbitol and rhamnose. The species is immunologically homogeneous except that freshly isolated strains contain two antigens, one of which is lost on continued cultivation, and antiserums prepared from stock strains may not agglutinate fresh strains too well. There is some cross-reaction, perhaps to one-quarter titer, with the Shiga bacillus, but agglutinins are not reciprocally absorbed. The Schmitz bacillus is related serologically to E. coli O112. Sh. dysenteriae type 2 has been found in Europe, India, the Sudan, and elsewhere. In the United States it is not as common as some of the other dysentery bacilli but is encountered with some frequency and implicated in extensive institutional and other outbreaks of dysentery,6,47 and as an important cause of dysentery among chimpanzees in captivity.35

Other serotypes. Strains of dysentery bacilli culturally identical with Sh. dysenteriae type 1 but immunologically unrelated were found by Dudgeon and Urquhart in Macedonia in 1919 and designated by them Bact. parashigae (-) in contrast to the Schmitz bacillus, which they termed Bact. parashigae (+). These

bacilli have been observed from time to time in various parts of the world, including the United States, associated with diarrheal disease. They were studied in some detail by Large and by Sachs and are sometimes known as the Large-Sachs group or Sachs group of dysentery bacilli. Sachs distinguished eight immunological types, but three of these were paracolon bacilli, leaving five valid types in all. The Sachs types are Q771, Q1167, Q1030, Q454, and Q902; these are now designated Sh. dysenteriae types 3, 4, 5, 6, and 7.

Other nonmannitol-fermenting dysentery bacilli which differ immunologically from type 1 have been described. Of these the organism designated *Sh. arabinotarda* type A and Gober and Stacy strain 8524 has been found to be identical with Q771, and *Sh. arabinotrada* type B with Q1167. Still another, *Sh. wakefield*, is a paracolon bacillus.

SHIGELLA FLEXNERI (GROUP B)

Soon after Shiga's discovery, Flexner, working in the Philippines, discovered other dysentery bacilli which for a time were not clearly differentiated. Flexner's bacilli and those described by Strong and Musgrave in 1900 differed from Sh. dysenteriae both serologically and in the fermentation of mannitol. Attempts to subdivide the bacilli of the Flexner group by biochemical methods have not been successful; a wide variety

may be separated on the basis of the variable fermentation of sucrose, dulcitol, sorbitol, maltose, raffinose, arabinose, inositol, and salicin, and indol formation, but such varieties are not correlated with immunological type and have had little practical value.

Shigella flexneri (Bacterium paradysenteriae, Pseudodysentery bacillus, Shigella paradysenteriae, Flexner's bacillus, Hiss and Russell's Y bacillus, Strong's bacillus).

Sh. flexneri is made up of a group of immunological types that are distinct and yet related to one another. Five immunological types were distinguished by Andrewes and Inman on the basis of the distribution of four antigens, V, W, X, and Z, which are designated as types V, W, X, Y, and Z. Subsequently Boyd reported evidence of the presence of type-specific and group-specific antigens in these types and in additional related forms and suggested that numbered types be substituted for the Andrewes and Inman types.

Further study¹⁴ has fully substantiated this view, and the commonly accepted serological types of *Sh. flexneri* are shown in the accompanying table. The nomenclature of these has become confused, since different designations for the same type have been used by various authors in various countries. The common synonymous designations are shown in this table, and the types are indi-

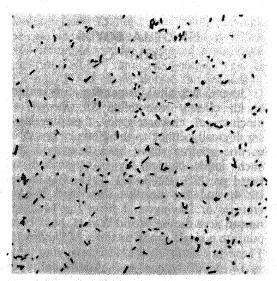


Figure 114. Shigella flexneri. Smear from a pure culture. Fuchsin; × 1050.

cated by Arabic numerals in accordance with the recommendation of the Shigella Commission to avoid confusion of Roman numerals with capital letters. As in the case of *Sh. dysenteriae*, the current Russian nomenclature differs from that of the International Schema; the correlations are shown in the accompanying table.

It may be noted here that certain nonmannitol-fermenting dysentery bacilli are closely related to the Sh. flexneri group on a serological basis and are, therefore, included in it. The Rio and Rabaul bacilli are Sh. flexneri type 4, but differ slightly in group antigens. The original Andrewes and Inman types are included in types 1, 2, and 3. Types 4 and 5 are the new types described by Boyd and originally called 103 and P119 respectively. Type 6, or Boyd 88, is not new but a variety of the Newcastle-Manchester bacillus that was first isolated from cases of dysentery in 1929. The X and Y types represent an as yet unsolved problem. These were so designated by Andrewes and Inman and apparently have no specific antigen; frequently cultures so labeled are found to be type 2, and in other instances specific agglutination may be masked by rough antigens.

Serological typing is readily carried out, and phage typing, useful with staphylococci and Salmonellas in particular, has not been of general interest. It has been reported³⁶ that preliminary investigation of phage sensitivities has indicated a close correlation with serological groups.

Colonial morphology, as accentuated by oblique transmitted light, appears to be associated with both virulence and antigenic content.⁹ Highly virulent colonial types contain a full complement of antigens, while morphologically distinct avirulent forms do not contain specific, or type, antigen, and are apparently transitional in the change from smooth to rough.

All of the Flexner bacilli are of world-wide distribution, the relative proportions varying from one locality to another. As a whole, Sh. flexneri is found in a large proportion of the dysentery cases in temperate climates, and even in tropical countries it is perhaps the most common of the dysentery bacilli. In this country, for example, 451 strains of a total of 769 isolated in routine examinations in Connecticut in 1940 to 1943 were Sh. flexneri, and of 1329 dysentery bacilli isolated from British and In-

Shigella flexneri and Shigella boydii

INTERN	ATIONA	L SCHEMA			OTHER NO	MENCLATUI	RE	<u> </u>
SPECIES NAME	SERO- TYPE	ANTIGENIC FORMULA	WHEELER	BOYD	ANDREWES- INMAN	GERMAN	RUSSIAN	OTHER
Sh. flexneri	1a	I: 3, 4, (7, 8)	I	V	v	B, C	$f(f_2)$	Flexner
·	16	I: (3, 4), 6	I		VZ	Α	$f(f_1)$	
	2a	II: 3, 4	Ha	W	W	D	c	
	2b	II: 7, 8	IIb		WX	DX	b	
	3a	III: 6, 7, 8	III		Z	H	е	
	3b	III: (3, 4), 6, 7, 8					d	
	3c 4a	III: (3, 4), 6 IV: 3,4	IV	103		102	$a(a_2); a(a_1)$	
	4a 4b	IV: (3, 4), 6	IV	103Z		F J	$a(a_2), a(a_1)$ $a(a_3)$	
	5	V: 7	V	P119		G	$g(g_1); g(g_2)$	
	6	VI: (3, 4)	VΙ	88		Ĺ	Newcastle	Sh. newcastle
			·			- -		Newcastle-
								Manchester bacillus
X variant		-: 7, 8	X					
Y variant		-: 3, 4	Y			Y1, Y2		Hiss-Russell
Sh. boydii	1			170			I	
	2			P288			V	
	3			D1		_ ::		
	4			P274		R	III	
	5			P143			VII	
	6 7			D19		N, P	II	Lavington
						14, 1	11	type T , Sh .
								etousae type 1296/7
	8						***	
	9						IV	
	10 11							
	12						VI	
	13						* *	
	14							
	15							

dian troops in India, 999 were Flexner, 197 Shiga, 100 Schmitz, and 33 Sonne bacilli.

Sh. flexneri forms no soluble toxin or exotoxin but contains an endotoxin. It has been studied in some detail by Goebel and his co-workers.37 who have found that the somatic antigen which contains the toxicity is made up of a lipid component, a protein component, two carbohydrate components one of which contains labile acetyl groups, and a toxic component which Goebel believes to be a distinct substance and possibly associated with a purine or pyrimidine-like substance. In general, it has not been possible to detoxify the endotoxin without destroying its antigenicity. Mucinase is produced but to lower titer than by the cholera vibrio,25 but whether this activity contributes to the pathogenicity of these microorganisms is not clear.

SHIGELLA BOYDII

(GROUP C)

mannitol-fermenting dvsenterv bacilli closely resembling Sh. flexneri in biochemical characteristics, but unrelated serologically either to the Flexner group or to one another, have been described. Six such immunological types described by Boyd as 170, P288, D1, D19, P143, and P274, and the bacillus described as Sh. etousae or Lavington I from the Mediterranean area during World War II, have been put into a single group as types of Sh. boydii. These are listed in the accompanying table as Sh. boydii types 1 to 7. Additional types have been described. Some of these are related to coliform bacilli as well as to some of the other Boyd types; specifically types 10 and 11 are related to E. coli O105 and to Sh. boydii type 4.

The pathogenicity of these forms appears to be closely similar to that of *Sh. flexneri*, and their distribution seems to be ubiquitous. They have not, however, been studied in detail, as have, for example, the Flexner bacilli, with respect to chemical characterization of endotoxin and somatic antigens, effective immunity, etc.

Escherichia alkalescens. E. alkalescens was described by Andrewes in 1918. Unlike the other dysentery bacilli, these bacilli ferment dulcitol. For some time they have been regarded as of uncertain pathogenicity, but evidence is accumulating which indicates that they are associated with disease. They have been found associated with sporadic enteric disease, and small epidemics are reported. In the last few years E. alkalescens has been recognized more and more frequently in this country and appears to be more common than had been supposed. It is also pathogenic for experimental animals.

Strains of E. alkalescens have been regarded as biochemically distinct and immunologically homogeneous. Stuart et al.,58 however, have found that the species is made up of a graded series of biochemical types. They have also demonstrated the presence of five antigens, two major antigens designated A and B, and three minor antigens, C, D, and E, in these bacilli. A, B, and C are present in all typical strains, while D and E occur singly or in combination to give four subtypes. They are immunologically related to the colon bacilli through the paracolon group and to Boyd P274. Two immunologically unrelated types have been described⁵⁴ and designated type II and type III in distinction to the original or type I. It has been suggested that type II be Sh. tieté. Both of these types include strains which ferment lactose.

There has been some tendency to put *E. alkalescens* together with *E. dispar* (see below) to make an alkalescens-dispar group of enteric bacilli set apart from the other Shigellas as more closely related to the coliform bacilli.¹⁸

SHIGELLA SONNEI (GROUP D)

Lactose-fermenting dysentery bacilli discovered by Duval in 1904 have since

been rediscovered by quite a number of observers. By force of usage these bacteria have come to be known as the Sonne type and are designated Sh. sonnei (Sonne-Duval bacillus, Duval's bacillus, Sonne's group III). The Sonne bacillus ferments mannitol and does not produce indol; it is serologically distinctive and homogeneous. There are two immunological types of Sh. sonnei which are designated I and II. Type I contains one antigen in predominance, while type II contains both antigens in equal amounts. The difference in agglutination titers is of practical importance, and type II antiserum is the one of choice. The two antigens differ in biochemical properties; that of type I is extracted from the bacilli in 50 per cent glycerol, while that of type II is not extracted in glycerol but in 7 M. urea. The rough form appears to be a variant of type II, but whether a succession of type $I \rightarrow type IIs \rightarrow type IIr occurs is not al$ together clear.55 Type I tends to predominate in the acute disease and is mouse-virulent, while type II occurs for the most part in carriers and is not virulent for mice.5

The lactose fermentation is slow and may be delayed for a week or 10 days, and strains of this type were doubtless confused with Flexner bacilli by earlier workers. It is probably considerably more common in the United States than appears in the records. It has also been reported in warmer countries, and perhaps a more detailed study of the differentiation of the mannite-fermenting bacilli would increase the amount of dysentery properly ascribable to this microorganism. ⁵⁹

The slow lactose fermentation would appear to relate *Sh. sonnei* to members of the colon group and in particular the so-called paracolon bacilli, but their immunological homogeneity tends to set them off.

Escherichia dispar. Other lactose-fermenting dysentery bacilli were designated Bact. dispar by Andrewes. Some of Andrewes' strains were Sh. sonnei, but the type now termed dispar differs from the Sonne type in that sorbitol is fermented and indol is formed. E. dispar is serologically heterogeneous and not related to Sh. sonnei but appears to be related to certain Flexner strains. It has been found that a group of 37 strains could be separated into three immunological types, two related to one another and the third independent. E. dispar may be divided into two varieties, E. dispar var. ceylonensis which ferments dulcitol, and E. dispar var. madampensis which does not.

BACILLARY DYSENTERY

Bacillary dysentery is a relatively common disease of man, more so in the warmer climates, and tends to be associated with crowding under conditions favoring spread of the infection from the human reservoir. It is more prevalent in backward countries, especially in the tropics and subtropics. It is a perennial problem in military operations, less so than in the pre-bacteriological days when, for example, more men died of diarrheal disease during the Civil War in this country than were killed in battle. As a seriously incapacitating disease tending to occur in epidemic form, it continued to be a problem in World War I when, for instance, it was a significant element in the outcome of the battle of Gallipoli; during World War II especially in the South Pacific and Mediterranean areas; and subsequently in situations such as the landing of American troops in Lebanon in the late 1950's. While the proportion of Shiga bacillus infections has sharply declined to reduce mortality, bacillary dysentery remains an important incapacitating disease.

The infection remains a local one in the bowel with invasion of little more than the superficial tissues, and the debilitating effects are attributable largely to the loss of fluid and electrolyte consequent to the purging diarrhea. There is no doubt of the etiological relation of the dysentery bacilli to the disease; in addition to the presence of enormous numbers in the acute disease, the immune response as serum agglutinin, and the production of analogous disease in certain experimental animals (see below), accidental infections of man are well known and the disease has been produced in human volunteers. ^{56, 57}

Pathogenicity for man. 65 The incubation period of bacillary dysentery is generally short, about 48 hours, and the disease may be acute or tend to run a chronic course. Apart from the inflammatory, sometimes ulcerative or diphtheritic, lesions in the intestine (ulcerative colitis), the anatomical picture of dysentery presents little that is characteristic. The liver abscesses that are found, as a rule, in amebic dysentery are

absent in the bacterial diseases, one series having been reported of 1130 cases of bacillary dysentery without a single abscess. Dysentery bacilli are sometimes found in immense numbers in the dejecta, often in almost pure culture. They may be found at autopsy in the mesenteric glands, but, as a rule, not in the spleen or other internal organs, nor do they commonly occur in the blood or urine. The pathogenesis of the disease in experimental animals (see below) - an inital penetration of the epithelial cells and the development of foci of infection in the lamina propria-may closely simulate the disease process in man. Bacillary dysentery is, therefore, not a septicemia but an infection localized in the alimentary tract, in this respect resembling cholera rather than typhoid fever. Recurrent diarrheal disease may be caused by dysentery bacilli, indicating that the bacilli may persist in the bowel, possibly in the superficial layers of the intestinal epithelium, for long periods. 11, 21

In the large series of cases studied in Denmark caused by the Sonne and Flexner types, the case fatality was about 2 per cent. The Shiga bacillus dysentery of the tropics is much more often fatal (20 per cent). The clinical disease is considerably more severe in the Shiga infections than in Flexner infections, and complications, such as arthritis, are almost invariably associated with the former type of infection.

Some portion, apparently an increasing portion, of infant diarrhea is of dysentery bacillus etiology. Certain kinds of bacillary dysentery, especially Sh. sonnei infections, have become more common, and the epidemiology of the disease has changed so that the disease seems to be shifting from "the scourge of armies to the bane of nurseries,"59 where it may assume a fulminating, rapidly fatal form.44 Some investigators have isolated the dysentery bacilli from the excreta, particularly in those cases in which there is mucus in the stools. Those cases with which dysentery bacilli are associated do not appear to differ clinically from those in which they are not found, and it is uncertain just what proportion of cases of infant diarrhea is caused by the dysentery bacilli.

Carriers. It is probable that dysentery bacillus infection is very common, many of the infections going unrecognized because of the mildness of the symptoms. Persons with such infections are, of course, con-

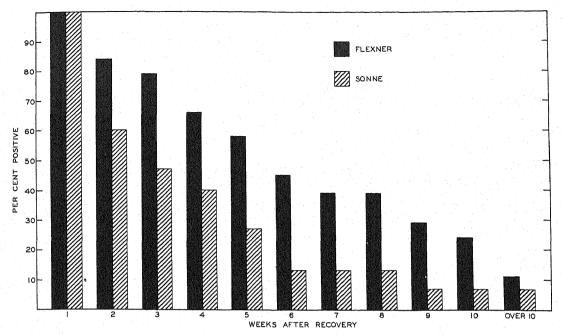


Figure 115. The persistence of dysentery bacilli in the feces of convalescents. (Prepared from data of Watt, Hardy, and DeCapito.⁶⁷)

valescent carriers who continue to discharge the bacilli for an average time of three to five weeks. It is not clear whether a permanent chronic carrier state analogous to the chronic typhoid bacillus carrier occurs, but many convalescents continue to discharge dysentery bacilli over long periods, and inapparent infection is not uncommon. In any case, it is the casual carrier and changing groups of casual carriers or ambulatory cases that are of primary importance in the maintenance and spread of the infection, and the disease persists in smoldering, endemic form. 40, 41

Pathogenicity for lower animals. The occurrence of bacillary dysentery in chimpanzees in captivity has been noted above, and other monkeys are commonly infected, usually with Sh. flexneri. It is probable that such infections are acquired from man and do not represent a natural infection in the wild state. The infection tends to be latent, with occasional appearance of symptoms, particularly when the animals are stressed.

A number of experimental infections have been described which vary in the degree to which they approximate the human disease. The fatal fulminating bacteremia produced in various experimental animals, such as the mouse, on intraperitoneal inocu-

lation is at best only distantly related to the human disease. An infection of the urinary bladder of the guinea pig may be produced by direct instillation of dysentery and certain other enteric bacilli,4 but it does not remain localized. The technique of inoculation of a ligated loop of the small bowel of the rabbit, devised by De and extensively used as an experimental model of cholera (Chap. Twenty-three), gives positive reactions, e.g., intraluminal fluid accumulation and bacterial multiplication, with dysentery bacilli considered to be virulent, but not with avirulent strains, e.g., Sonne II.1.61 A keratoconjunctivitis may be produced in the guinea pig eye by instillation of the bacilli, and this infection the histopathology closely resembles that found in experimental enteric infections. 50

Enteric infections, produced by oral administration of dysentery bacilli, have been studied in mice^{10, 51} and have been produced in the guinea pig with streptomycin-resistant bacilli administered in a streptomycin-containing inoculum and/or following pretreatment with the antibiotic,³⁴ or following parenteral inoculation of carbon tetrachloride.^{26, 27} Production of such infections also requires starvation and inhibition of peristalsis with laudanum or morphine. The experimental enteric infection most closely

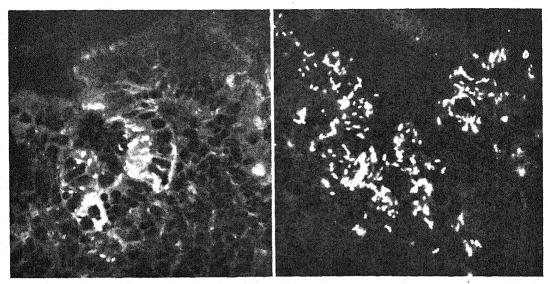


Figure 116. The pathogenesis of bacillary dysentery. Left, penetration of the ileal epithelium in the guinea pig at 12 hours by Sh. flexneri 2a; bacilli can be seen in the epithelial cells and some in the lamina propria. Right, small micro-ulcer in the colonic epithelium in the monkey 24 hours after infection; bacilli are present in the epithelial cells and in the lamina propria of the tubular glands of the colon. (LeBrec.) (Figure at right, courtesy of Journal of Bacteriology.)

simulating the human disease is that in the monkey, usually rhesus, which, as just indicated is also acquired naturally from man, and does not depend upon pretreatment to break down resistance.

Pathogenesis. Within the past few years the pathogenesis of bacillary dysentery and the nature of the virulence of dysentery bacilli have been clarified through the studies of Formal, LaBrec, and their co-workers. In essence, there are two stages in the former: penetration of the epithelial barrier by direct penetration of epithelial cells and into the lamina propria, and multiplication in the lamina propria as foci of infection. The subsequent breakdown of tissue with ulcer formation is due to endotoxin formed in situ rather than to the absorption of this, or other, toxin from the lumen of the bowel. Penetration of the epithelial barrier and the development of foci of infection in the deeper tissues has been demonstrated by the fluorescent antibody staining technique in the guinea pig48 and in the monkey.49 This occurs in the colon in the monkey, and in the guinea pig first in the small bowel as a consequence of opium-induced stasis and subsequently in the large bowel.28

In a series of elegant experiments resting on the genetic basis of virulence and genetic homology between dysentery bacilli and E. coli K-12, it was shown that penetration of epithelial cells and ability to multiply in the lamina propria are separable components of virulence. Avirulent variants were found to be unable to penetrate the epithelial barrier, remaining within the lumen of the bowel,²⁴ and full virulence could be restored to some variant strains by mating with *E. coli* K-12, supporting the assumption that the virulent phenotype is a polygenic phenomenon.²⁹ Conversely, hybrids of virulent Shigella and Escherichia are able to penetrate epithelial cells to give an inflammatory reaction in the lamina propria, but an abortive infection ensues since the hybrid does not multiply to produce a focus of infection.³⁰

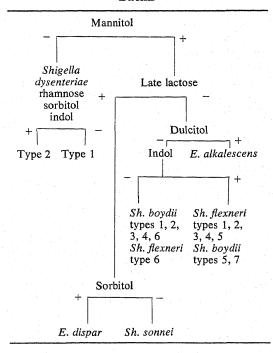
diagnosis.45 Since, **Bacteriological** indicated earlier, dysentery is a clinical rather than an etiological entity, the causative microorganism must be isolated and identified to allow a diagnosis of bacillary dysentery. The bacilli may be found in fecal specimens, and rectal swabs are cultured for the detection of carriers. The methods of enrichment culture and direct plating are essentially those used in the isolation of Salmonella and the typhoid bacillus. Desoxycholate-citrate and S-S agars are the most useful of the agar mediums; bismuthsulfite is not suitable because species other than Sh. flexneri are inhibited. Colonies are subcultured and identified by biochemical reactions and slide agglutination. For tentative serological identification polyvalent serums may be used, including (1) polyvalent Shiga serum, (2) polyvalent Flexner serum, (3) polyvalent Sh. boydii serum, (4) Sh. sonnei types I and II, (5) E. dispar types, and (6) E. alkalescens. The cross-reaction between these groups of antiserums may be removed by agglutinin absorption.13 For precise identification monospecific antiserums are required. The possibility of rapid presumptive diagnosis by identification of the four groups of Shigella in direct fecal smears stained with fluorescent antibody has been of considerable interest. It appears to be of value in Sonne infections, but false positive reactions tend to occur in Flexner infections.63

Chemotherapy. 46, 62 The sulfonamides are highly effective chemotherapeutic agents in bacillary dysentery and have been widely used, both for therapy and prophylaxis. The soluble compounds, such as sulfadiazine, are more effective than the nonabsorbable compounds. Streptomycin and the broadspectrum antibiotics, tetracycline and chloramphenicol, are also effective, as is furazolidone (Furoxone), and all of these also have been of interest as chemoprophylactic agents.

With the general use of sulfonamides, sulfonamide-resistant strains of dysentery bacilli became more and more common, resulting in increased usage of the other antimicrobial agents, with the consequence that multiple resistant strains were encountered more and more frequently. In 1960 it was found in Japan⁶⁴ that the multiple resistance (sulfonadmide-streptomycintetracycline-chloramphenicol) acquired by sensitive strains of Shigella, as well as other enteric bacilli, is episome-mediated and transferred in toto. The episome is designated the R-factor (Chap. Seven), and this kind of acquired drug resistance has been found to occur world-wide. Effective chemotherapy of bacillary dysentery, then, continues to offer practical difficulties.

Epidemiology. Infections caused by dysentery bacilli are probably far more common than is generally recognized. In 1945, 33,495 cases and 400 deaths were reported from 38 states, rates of 32.4 and 0.4 per 100,000 respectively. Attacks of severe illness grade off into mild and almost trivial attacks of simple diarrhea. Several outbreaks of typical "food poisoning" attributed to the Sonne bacillus are on record.

Biochemical Separation of the Dysentery Bacilli



In a number of localities where the careful bacteriological studies have been made, dysentery bacilli have been found widely distributed both in patients with gastrointestinal derangements and in the general population. Probably the most important single reservoir of infection is the human carrier, either convalescent or with inapparent infection. The extent to which the carrier state occurs has been appreciated only in recent years. In a study of dysentery bacillus infection in the normal population Watt and Hardy66 found Sh. flexneri in 11 per cent of the population in New Mexico, 4 per cent in Puerto Rico, 3 per cent in Georgia, and 0.1 per cent in New York City, with an estimated annual morbidity of 60 per cent in Puerto Rico, 48 per cent in New Mexico, and 20 per cent in Georgia, and an over-all ratio of convalescent or passive carriers to cases of 9:1.

Dysenteric infections seem to be most common in hot countries and in the summer months in temperate climates, although they may occur at any season of the year. The acute diarrheal disease occurring routinely in tropical countries, and known locally as "Gyppy tummy," "Delhi belly," etc., are frequently bacillary dysentery, the Flexner and Boyd types appearing to

be the most common where studies have been made.22,23 The spread of the disease is due to the more or less direct transfer of the specific bacillus from infected intestinal discharges to the alimentary tract of a fresh individual. Polluted water may play a part in some outbreaks but is apparently not nearly so important a factor in dysentery as it is in typhoid fever. Improper disposal of excreta permitting dissemination by flies, and the contamination of food by chronic carriers and convalescents, appear to be the most important factors in the spread of bacillary dysentery. The role of insects, especially flies, has been demonstrated,40 and is probably an important one, and the seasonal incidence of bacillary dysentery is in keeping with this. In the epidemic reported by Kuhns and Anderson infected flies were caught in kitchens and operating latrines; similar reports are not uncommon. The decline in diarrhea and enteritis and the shifting proportions of the etiologic agents of the last few decades are very likely a reflection of the general improvement in sanitary conditions. 60

At the present time in temperate climates dysentery flourishes especially in mental

hospitals and other large institutions, where lack of personal hygiene among the inmates favors the transfer of infection. Whenever it gains a foothold in these institutions it seems to be kept alive chiefly by chronic carriers and proves an obstinate problem. Weekly bacteriological examination in one institution showed that more than 50 per cent of the dysentery patients continued to excrete dysentery bacilli for long periods in one case over four and one-half years. Epidemic bacillary dysentery is also a disease of armies in the field, where opportunities for the dissemination of infection are frequently very great, and extensive outbreaks are common.

Although in small outbreaks a single type of dysentery bacillus may be found, in the larger outbreaks more than one type is almost always observed. The most common types in this country are the Flexner (often type 2a) and Sonne bacilli. The proportions differ from one area to another and with the season. In the fourth quarter of 1966, for example, the Flexner type occurred in 70 per cent and the Sonne type in 28 per cent of cases in California, and 58 and 40 per cent respectively in Florida. In

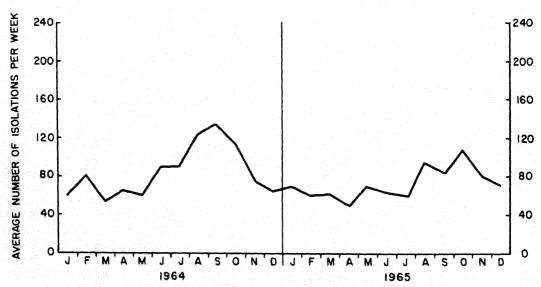


Figure 117. The frequency of isolation of dysentery bacilli from man in 17 states of the United States in 1964 and 1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

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the southern half of the continental United States the occurrence was Flexner bacilli 75 per cent and Sonne bacilli 23 per cent, and in the northern half the percentages were 40 and 60 respectively. The seasonal rise in late summer and early fall is largely accounted for by increases in the number of Flexner bacilli infections.

Immunity. The development of an immunity effective to some degree is indicated by the relative resistance of the resident population of an endemic area to the acute disease which affects recent arrivals, e.g., "acclimatization diarrhea." This phenomenon is well known to residents of temperate climates visiting tropical and subtropical areas.

Antibodies, agglutinins, are formed in response to infection with dysentery bacilli, usually appearing after the sixth day. The titer is relatively low as a rule. The diagnostic significance of agglutinins is somewhat uncertain largely because "normal" agglutinins are common. Normal serum commonly agglutinates Sh. dysenteriae 1 in 1:20 dilution, but a titer of 1:40 or higher is suggestive of infection. Agglutinins for Sh. flexneri occur in much higher titer, as high as 1:150 in normal serum, and the titer is frequently increased in infections with other species of dysentery bacilli. Flexner agglutinin has, then, very little diagnostic value unless it is to high titer, and agglutinins for Shiga and typhoid bacilli are absent. Agglutinins to the Newcastle bacillus, Sh. dysenteriae 2, and E. alkalescens occur only in low titer. Sh. sonnei is often agglutinated to titers as high as 1:50 by normal serum, and, though the titer may occasionally be very high in infection, sometimes little or no agglutinin response is apparent.

The antibody demonstrable as agglutinin, and as protective antibody by the mouse protection test, is apparently unrelated to effective immunity. Consistent with this, vaccine prophylaxis of bacillary dysentery has been almost uniformly ineffective. In this connection it may be recalled that the infection is confined to the lumen of the intestine, intestinal mucosa, and lymphoid tissue as a rule, and in a very real sense remains outside the body. Coproantibody has been demonstrated in bacillary dysentery, but its part, if any, in effective immunity is uncertain.

Unlike infections with typhoid and related bacilli, bacillary dysentery remains a localized infection, and the bacilli rarely penetrate the body beyond the regional lymphatics at the most. An effective immunity is, therefore, one functional at the local level, and present evidence suggests that an essential feature is a stimulation of the immune response in the antibody-forming cells of the lamina propria. Such an immune response is most effectively produced by the local application of antigen, e.g., vaccine by the oral route. The use of killed vaccines in this way has given equivocal results, but a considerable degree of effective immunity is produced by living vaccines.

Two kinds of living vaccines, administered by the oral route, have been used. One consists of bacilli made streptomycindependent and therefore unable to multiply in the absence of the antibiotic. Such vaccines of *Sh. flexneri* have been field-tested in Yugoslavia, and have been found to give significant, but type-specific, protection against the naturally occurring infection.⁵²

The other kind of vaccine is based upon studies of the pathogenesis of the disease and the virulence of the bacilli described above. Two types of vaccine have been prepared with Sh. flexneri 2a and tested in monkeys. The one is an avirulent mutant characterized by inability to penetrate the epithelial barrier, and the other a hybrid strain capable of penetrating epithelial cells but unable to maintain itself to produce foci of infection in the lamina propria. Both have proved to be highly effective in this model in preventing the development of disease in response to challenge inoculation with the virulent parent strain.31 In such immunized animals the virulent strain is unable to penetrate the epithelial barrier and remains in the lumen of the bowel.33 Polyvalent vaccines of hybrid strains of Sh. flexneri 1b, 2a, and 3 and Sh. sonnei I given in two doses by the oral route have been shown to produce a highly effective immunity against challenge with the virulent parent strains.32 The type specificity of the immunity so produced is pointed up by the observed lack of protection against Sh. flexneri 6 challenge, a strain not included in the vaccine.

The type-specificity of the immunity produced by such vaccines would appear to be a limiting factor in their utilization in view of the multiplicity of dysentery bacilli. Almost invariably, however, bacillary dys-

entery in a given area is caused by two or three kinds of bacilli, usually types of *Sh. flexneri* and *Sh. sonnei*. In practice, therefore, immunization against two or three kinds of dysentery bacilli should suffice to control a very large portion of naturally acquired infections.

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Chapter Twenty-three

THE CHOLERA VIBRIO AND RELATED FORMS

Although Asiatic cholera has doubtless smoldered endemically in parts of India for many centuries, the year 1817 marked its first considerable extension beyond the borders of that country. Europe was first invaded in 1831, and since that date a series of great epidemics has carried the disease over a large part of the civilized world. The disease was brought to New York by Irish immigrants during the pandemic of 1832-33 and in the pandemic of 1846-62 invaded the United States via New Orleans (1848) and spread up the Mississippi Valley. The fourth great pandemic, that of 1864-75, affected Asia, Africa, Europe, and America. 125 The causal agent of the disease, the cholera vibrio, was discovered in 1883 by Koch in the intestinal discharges of cholera patients. Similar microorganisms were described by later workers in infected water and elsewhere, and now a number of species are known. In general, however, these other vibrios are nonpathogenic and have been studied in terms of their relation to the cholera vibrio and are, therefore, not particularly well known. Of what is now a fairly sizable group, only a few species are pathogenic: the microorganism discovered by Koch and variously termed Spirillum cholerae asiaticae (Koch), S. cholerae, Vibrio cholerae, the comma bacillus and V. comma (Bergey); certain of the El Tor vibrios; Vibrio fetus, infecting domestic animals; and a vibrio pathogenic for pigeons and guinea pigs, Vibrio metchnikovii.

VIBRIO CHOLERAE41, 119

Morphology and staining. The cholera vibrio is a short, slightly curved and twisted

rod, 1.5 to 3 μ in length and 0.4 to 0.6 μ in breadth. It may occur singly or in chains which have the appearance of short spirals or S-shaped forms (two cells). The straight and spiral threads formed in the pellicle of liquid gelatin cultures are usually regarded as involution forms. Cultures that have been maintained for a long time on agar often lose the curved form and appear as straight rods but resume the more characteristic form when passed through animals. The vibrios are actively motile by a single polar flagellum which is shorter than the flagella of most bacteria. Spores are not formed. The cholera vibrio stains readily with the ordinary aniline dyes and is gram-negative.

Colonies on agar mediums are similar to those of the other enteric bacilli but may be distinguished from those of *Escherichia coli* by their thin, opalescent appearance. They are 1 to 2 mm. in diameter, low, convex, and grayish yellow in color, with a finely granular consistency which is accentuated under low magnification. Some strains are hemolytic on blood agar while others are not (see below).

Physiology. The cholera vibrio is strongly aerobic, and only very sparse growth appears under anaerobic conditions, and then on prolonged incubation. It grows over the temperature range of 16° to 42° C. with optimum growth at 37° C. An alkaline reaction is essential for good growth; the bacteria will grow over a pH range of 6.4 to 9.6 and are usually cultivated at an alkaline reaction, pH 7.8 to 8.0. This marked tolerance for alkali is taken advantage of in the preparation of selective mediums for the isolation of the cholera vibrio, the pH of such mediums usually being about 9.5. They are not nutritionally fastidious and may be

grown in peptone water. Many strains grow on simple synthetic mediums¹³² containing ammonium sulfate as a source of nitrogen, but some require added purines.⁶

The relative resistance of the cholera vibrio, as a gram-negative enteric form, to inhibitory substances such as bile salt, bismuth-sulfite, and tellurite is taken advantage of in the preparation of a variety of selective mediums, liquid for purposes of enrichment and solid for isolation^{100, 113} (see below).

Fermentation reactions are variable, and a number of carbohydrates, including dextrose, levulose, galactose, maltose, sucrose, and mannitol, may be fermented with the production of acid but no gas. Lactose, inulin, and dulcitol are not attacked. Heiberg studied the fermentation reactions of the vibrios at some length and arrived at six fermentative types on the basis of the fermentation of sucrose, arabinose, and mannose which are known as the Heiberg types. Type I is characterized by the fermentation of sucrose and mannose but not arabinose, and contains all of the cholera vibrios and some noncholera varieties. Starch is hydrolyzed.

Both coagulated serum and gelatin are liquefied. Stab cultures in gelatin often develop a small turnip-shaped area of liquefaction at the surface, which by evaporation of the fluid leaves a bubble-like depression. Other vibrios besides the cholera vibrio

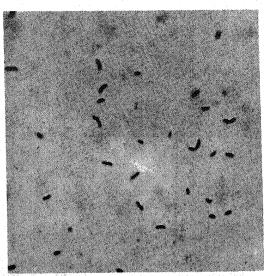


Figure 118. Vibrio cholerae; pure culture in peptone water. Gram stain; × 1200.

The Heiberg Fermentation Types of Vibrios

SUGAR	ТҮРЕ					
	I	II	III	IV	V	VI
Sucrose	1 +	+	+	+	-	
Mannose	+	_	+	_	+	
Arabinose	_		+	+	_	

produce this same type of liquefaction. Growth in milk does not produce any visible change for some time, but a slow peptonization without coagulation appears on continued incubation. Hydrogen sulfide and indol are produced, and nitrates are reduced to nitrities. The addition of sulfuric acid to a culture of the cholera vibrio in nitrate-peptone broth results in the development of a red color—the so-called cholera-red reaction. This is the nitroso-indol reaction and is given by any bacterium, the colon bacillus for example, that both reduces nitrate and produces indol. Other vibrios give this reaction also.

The resistance of the cholera vibrio to various injurious influences is not great. It is killed by moderately high temperatures (10 minutes at 55° C.) and is destroyed quickly by chemical disinfectants. It is particularly sensitive to drying; if a drop of broth culture be dried on a slide, the vibrios are all dead in about two hours. It does not survive long in association with the ordinary saprophytic bacilli of soil and water, and whether it is able to multiply outside the body in impure water is uncertain. Upon the surface of vegetables and fruits kept in a cool, moist place, the vibrios may remain viable for from four to seven days.39 The slight resistance of the cholera vibrio, especially its sensitiveness to drying, explains the rapid and complete disappearance of cholera in once infected localities and also the circumstance that the disease is rarely, if ever, airborne.

Cholera vibrio biotypes. The foregoing description is that of the "classic" cholera vibrio as isolated by Koch and persisting until relatively recently as the predominant, if not exclusive, cause of cholera. Particularly within the last decade it has become clear that, although the general character of the cholera vibrios as described above holds true, causative vibrios occur as a number of differentiable biotypes.



Figure 119. Flagella of vibrios. Left, Vibrio cholerae; right, a paracholera vibrio. × 2000. (Kral.)

At the turn of the century vibrios were isolated from persons passing through the quarantine station at Tor, some of which were immunologically indistinguishable from *V. cholerae* (see below) but differed in that they produced a soluble hemolysin. These so-called El Tor vibrios were again thoroughly studied at Tor in 1930 and 1931 by Doorenbos. The practical quarantine problem was answered by the general acceptance of their nonpathogenicity.

The question of the pathogenicity of the hemolytic vibrios was raised again by the occurrence of an epidemic of clinical cholera in Celebes in 1937 and 1938. 93, 102, 133 About

400 strains of the vibrio were isolated and found to be immunologically identical with the cholera vibrio but hemolytic, and were termed the Celebes vibrios. Cholera caused by these, or closely similar, hemolytic vibrios recurred from time to time in Indonesia in the 1940's, and by 1961–62 had spread widely in the southwest Pacific area (see below). The consequent greatly increased interest in cholera resulted in much more detailed studies of the vibrios and the development of a number of differential characteristics separating the cholera vibrios into biotypes.

Particular interest has centered about the

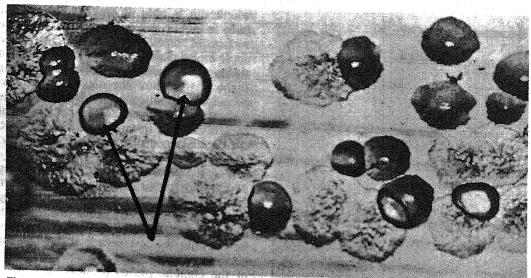


Figure 120. The colonial morphology of *Vibrio cholerae* on bile salt agar in mixed culture with coliform bacilli. The minute, translucent, raised colonies of the cholera vibrio contrast with the roughened appearance of coliform bacillus colonies in the presence of taurocholate. \times 10.

TOXÌNS 533

hemolytic activity, and at present soluble hemolysin and blood plate hemolysis are distinguished. The test for the former consists essentially in the incubation of broth culture of the vibrios with a suspension of sheep or goat erythrocytes and, as originally applied, was known as the Greig test. There are a number of variables, e.g., the requirement for calcium, the inhibitory effect of sodium chloride, erythrocyte species, and incubation times and temperatures, and the conditions affecting the test have been studied and a standard method described.36 Blood plate hemolysis is of the β -type, and some strains produce a discoloration known as hemodigestion. The other characteristics differentiating the biotypes are sensitivity to type IV phage, 106 chicken cell agglutination,47 the Voges-Proskauer reaction, and polymyxin B sensitivity. 72, 126 The last is determined with 50 unit discs on nutrient agar: classic cholera strains are sensitive and the so-called El Tor stains resistant.

On the basis of such characterization, a number of biotypes occur^{25, 27, 28} with resulting confusion in nomenclature. Feelev³⁵ has introduced a system with the definition of biotypes designated by number, and these are shown in the accompanying table. It is evident that the occurrence of intermediates between the "classic" and El Tor vibrios has removed the distinction from these terms. though they continue to enjoy some popular usage in which vibrios other than the "classic" types are called El Tor, and the biotypes are considered to be varieties of V. cholerae. As yet the epidemiological significance of such biotypes appears to be limited to a general differentiation of the "classic" type from the others, and the stability of the

differential characteristics remains somewhat uncertain.^{28, 64, 109, 127}

Toxins. Unlike the dysentery bacilli, the cholera vibrio remains within the lumen of the bowel, with no evidence of penetration of the epithelial barrier. This is taken to indicate that the disease is essentially an intoxication, a supposition which has been supported by the demonstration that, under experimental conditions, the manifestations of infection may be wholly accounted for by the activity of cell-free toxin. 10 The question of the absorption of toxic substances remains largely unresolved although in the opinion of many workers23 the observed histopathology is not entirely attributable to dehydration and acidosis characteristic of the disease (see below). Interest has centered largely about the local manifestations of the disease, and the presumably local effects of toxin, in the altered water and ion movement resulting in the purging diarrhea and concomitant loss of fluid and electrolyte.

The cholera vibrios form a multiplicity of toxins, some of which are separable into groups, or types, on the basis of their occurrence in the cell and in culture, their heat stability, and their dialyzability. The toxic action of each type is demonstrable in more than one way, and it is not yet clear whether this is an artifact of assay methods, or whether differentiable toxins occur within each type. These toxins are summarized in the accompanying table.⁹

Type 1 toxin is apparently endotoxin of the Boivin type, *i.e.*, extractable in trichloracetic acid solutions, glycols, etc., and acts as a lethal toxin in the mouse and chick embryo. There seems to be no evidence that

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Cholera Vibrio Biotypes (Feeley)

ТҮРЕ	HEMOLYSIS		PHAGE IV SENSITIVITY		CHICKEN CELL AGGLUTINATION	Voges- Proskauer
	TUBE	PLATE				
1*	-			+	-	±
. 2				+		
3†		+				+
5		+			+	+ 4

^{*&}quot;Classic" cholera vibrio.

[†]El Tor vibrio.

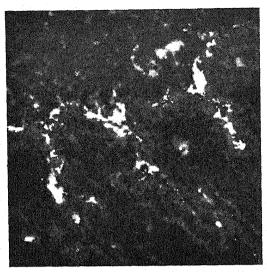


Figure 121. Fluorescent antibody-stained frozen section of the small bowel of a guinea pig infected with cholera. The vibrios are apparent in the lumen, sticking to the epithelial cells at the villus tips, extending into the crypt areas, and filling indentations of the epithelium. Failure to penetrate the epithelium is evident. (LaBrec.)

it is active at the local level in the bowel, but it may be absorbed as a consequence of permeability alterations by other toxins to contribute in an as yet undefined way to the pathogenesis of the disease.⁷⁷

The other two types of toxin appear to be active at the local level. The dialyzable heat-stable toxin of type 3 markedly inhibits the active transport of sodium in anurian epithelium in vitro^{57, 73} and in vivo.⁸⁹ The hypothesis that such inhibition of the sodium

pump is responsible for the diarrhea is an attractive one, and has been strongly advocated. 114 It has been found, however, that type 3 toxin freed of other toxins as a dialysate does not induce the required changes in water and ion movement in experimental models, 15, 89 a finding that negates its primary role in the pathogenesis of the disease.

In contrast the toxins of type 2, the socalled cholerigenic toxins, produce water and electrolyte loss - as in diarrhea in the infant rabbit and intraluminal fluid accumulation in the ligated ileal loop of the rabbit (see below) - in crude form or when separated in purified form from toxins of types 1 and 3. The activity is exotoxic in that it is found in the liquid culture supernatants. As assayed in the infant rabbit, type 2 toxin has been termed choleragen (formerly procholeragen A).48,49 The composition of the intraluminal fluid in the rabbit ileal loop closely resembles that of the human cholera stool,90 and liquid culture supernatants have been shown to produce cholera-like disease in man.4 A toxin of similar properties, designated the permeability factor, produces an intradermal reaction characterized by an increased capillary permeability19,20 resembling that observed in the bowel tissue in the human disease. Type 2 toxin is also demonstrable by cytopathic effects, swelling, accumulation of intracellular lipid, detachment, and lysis on mammalian cells in culture. 120 This toxin, or group of toxins, is produced by the "classic" and other biotypes of the vibrio, but relatively few strains are found to be highly toxigenic in vitro.

Cholera Toxins

TYPE	BIOLOGICAL ACTIVITY		OCCURRENCE			DIALYSIS	
		CELL WALL	INTRACELLULAR	SUPERNATANT	HEAT		
1*	Mouse lethal Chick embryo lethal	+ +	+ + +	_	Stable	Nondialyzable	
2†	Rabbit ileal loop Infant rabbit Intradermal Cell culture		+ + + + + + + + + + + + + + + + + + + +	+ + +	Labile "	Nondialyzable	
3‡	Anurian epithelium Kidney PAH uptake	+ +	.	+	Stable	Dialyzable	

^{*}Endotoxin.

^{†&}quot;Cholerigenic" toxins.

[‡]Active transport inhibitors.

Hemolysin. The hemolysin produced by some biotypes of *V. cholerae* occurs, together with other toxins, in liquid culture supernatants.¹³⁷ It has been studied in highly purified form¹³⁸ and may be a lecithinase.⁹⁴ It is apparently unrelated to the pathogenesis of the disease produced by these microorganisms,¹³⁹ but the occurrence of antihemolysin in patients' serms may be a useful serological method for retrospective diagnosis.⁴³

Mucinase. The cholera vibrio and related forms also produce mucinase activity which desquamates the intestinal epithelium of the guinea pig in vitro and is titrated in vitro against ovomucin. 16, 78 More than one serologically different mucinase is produced by V. cholerae, and potent mucinases are produced by noncholera vibrios, so that the relation of this activity to the pathogenesis of the disease is doubtful. 53

Antigenic structure. The first demonstration of bacterial agglutination with homologous immune serum by Gruber and Durham in 1896 was the agglutination of the cholera vibrio and the typhoid bacillus. It was soon apparent that the agglutination reaction was specific, and it has been used for the identification of the cholera vibrio since. The antigenic structure of the cholera and related vibrios has, therefore, been of considerable interest in connection with the differentiation of *V. cholerae*.

The vibrios contain both somatic heatstable O antigens and flagellar heat-labile H antigens. The type-specific antigens are O antigens, and a number of serological groups have been established; the cholera vibrio and certain of the El Tor vibrios (see below) fall into that designated O group I.

It was first shown by the Japanese workers that three serological types of the cholera vibrio may be differentiated on the basis of the specificity of the heat-stable O antigens. These are the "original," J, or Inaba type;

the Hikojima, "middle", or "intermediate" type; and the "variant," F, or Ogawa type. For many years only the Inaba type was found in India, but now the Ogawa type is often observed. The Hikojima type appears to occur primarily in China together with the Ogawa type and Inaba type. Some have questioned the validity of the Hikojima type, but there is no doubt that it occurs. 60 The distribution of these types has no epidemiological significance, at least in India, and, in fact, their stability is uncertain. Shrivastava and White¹²⁹ found that cultivation in the presence of heterologous antiserum results in conversion of Ogawa strains to Inaba, and of some Inaba strains to Ogawa. Other workers⁵ have found, however, that the change seems to occur in only one direction; i.e., cultures of the Ogawa serotype tend to throw off Inaba variants.

A detailed analysis of the O and H antigenic structure of the cholera and related vibrios showed that a group-specific O antigen, designated A, is shared by all vibrios of group I, and that the Japanese types are determined by subsidiary O antigens, B and C, and are arbitrarily designated type-specific. It was suggested that the Japanese types be indicated by their antigenic formulas, i.e., Inaba as type AC, Ogawa as type AB, and Hikojima as type ABC. Vibrios containing the group antigen but lacking these type-specific antigens have been found and thus constitute a new type. Additional antigens making up a variety of serotypes have been described. 14, 58, 134 The occurrence of certain antigenic types has been questioned but the low titer antiserums used prevented their demonstration: type A, for example, has been observed among strains isolated in Bangkok in 1958-1959. Antigens other than the group- and type-specific antigens are demonstrable also by the application of other methods of assay such as gel diffusion.67, 68, 99 The H antigens, designated by Arabic numerals,

Serological Types of Cholera and Paracholera Vibrios

	JAPANESE TYPE			
NAME	SYNONYMS	IMMUNOLOC TYPE	FICAL	
None Inaba Ogawa Hikojima	None "J," Japonica 1911, "original," "end type" "F," Formosicana 1911, "variant," "end type" "Middle type"	Type A Type AC Type AB Type ABC	Logical Section (1997) Section (1997) Section (1997)	

and other components of the O antigen are shared by the O group I vibrios with vibrios of other O groups.

Antigens other than those demonstrable by the conventional agglutination-agglutinin absorption method of antigenic analysis were first observed as giving rise to protective antibody effective in the mouse and guinea pig enteric infection assay and as producing antibody demonstrable in the passive hemagglutination reaction and by complement-fixation.¹¹ Gallut⁶¹ separated this antigenic complex as a precipitating antigen by phenol extraction of specific polysaccharide-containing antigens. This complex, designated nonagglutinogen antigen, or NAA, is shared in part with non-cholera vibrios.

A curious terminology has crept into the cholera literature with respect to agglutination; it is said that a strain which is agglutinated by specific antiserum is "agglutinable" while those vibrios which are not agglutinated by cholera antiserum are called "inagglutinable" despite the fact that they are readily agglutinated by homologous antiserum. These are referred to as the nonagglutinating vibrios, or NAG vibrios.

The cholera vibrios are serologically related to Brucella, and this has given rise to positive Brucella agglutination tests in persons who are not infected with Brucella but have been immunized with cholera vaccine. Gallut⁵⁹ has shown that the common antigens are the C and D antigens of the cholera O antigenic complex. An antigenic relationship of certain vibrio strains to coliform bacilli of the Bethesda group has been described also.⁹⁵

Variation. The cholera vibrio is well known for its tendency to develop bizarre involution forms. These are found not only in old cultures and cultures grown under somewhat adverse conditions, such as increased salt concentrations, but are also produced by the inclusion of substances such as glycine or alanine in the medium. Such changes in morphology are also associated with the usual type of S-R dissociation which occurs readily under the influence of bacteriophage, lithium chloride, etc., the rough variants showing distinctive colonial character, spontaneous agglutination in salt solution, and so on.

The immunological changes associated with the S-R dissociation have been studied in some detail by White. 141 He has found

that four groups of rough variants are immunologically distinguishable, and his group A appears to correspond to the group I of Gardner and Venkatraman. Further degeneration to the so-called ρ variants results in a loss of specific O antigen. An independent type of variation, rugose-nonrugose, may be induced, and the rugose strains of S, R, and ρ variants contain an O antigen that is common both to members of group A and to vibrios of other groups. A somatic protein antigen has been isolated by White which is common to all known variants and shows wide cross-precipitin reactions throughout the vibrio group, although it appears not to be concerned in the agglutination reaction.

Classification. While the cholera vibrio is set apart taxonomically from the Enterobacteriaceae as a genus of the tribe Spirillaceae, it is often, perhaps subconsciously, considered with the enteric bacilli. Doubtless this is attributable largely to the kind of disease it produces, but the cholera vibrio does resemble the anaerogenic enteric bacilli, such as Shigella, by its fermentation of glucose via the glycolytic pathway, by its having sufficient homology to share the R-factor with enteric bacilli. 85 etc.

Classification at this level has not been a matter of great concern, but both theoretical and practical considerations have entered into the characterization and nomenclature of the cholera vibrios. The name Vibrio comma of the Bergey classification has never gained acceptance outside this country, and cholera has occurred exclusively elsewhere for many years. The name Vibrio cholerae is almost universally used, and is now internationally accepted. 122

The status of the biotypes described above has not yet been formally settled. The separation of Feeley's type 3 (see above) as a different species, *Vibrio eltor*, is advocated by some, but is considered by others to be no more than a biotype⁷⁴ or variant³⁸ of *V. cholerae*. It seems probable that the latter view will prevail.

Pathogenicity for man.^{23, 86} The causal connection between cholera and the microorganism discovered by Koch has been demonstrated by a number of laboratory accidents. One of the first occurred in Koch's laboratory, and other infections resulting from the accidental swallowing of cultures of the cholera vibrio have been noted since. In one instance the swallowing

was deliberate; Pettenkofer and Emmerich voluntarily swallowed a small quantity of broth culture of "Koch's vibrio" and as a result developed cholera.

As indicated above, substantially identical clinical disease is produced by the several biotypes of V. cholerae. In addition, other unrelated vibrios are also associated with diarrheal disease. $^{97, 98}$ Such vibrios gave a positive reaction in the infant rabbit, and others, including certain water vibrios, initially unable to infect the infant rabbit, did so after animal passage. 32

Both laboratory cases of cholera and those cases contracted naturally in the course of epidemics are marked by great differences in the susceptibility of different individuals. The possible relation of the malabsorption syndrome, with leaf rather than finger patterns of villi, the amount of hydrochloric acid in the stomach, the diet, and similar factors have all been of interest in this connection, but as yet none has been clearly associated with susceptibility to infection.

The incubation period is short; it is usually given as three to five days but may occasionally be as short as 24 hours. The sequence of events appears to be as follows: The vibrios pass the barrier of gastric acidity in sufficient numbers to set up a focus of infection in the small bowel. Toxin, presumably the type 2 toxin described above, is elaborated, and fluid and electrolytes are rapidly lost in the resulting purging diarrhea. 115 This initial reaction is sometimes referred to as the first stage of the disease. The rice water stool is innocuous in odor and appearance; is a transudate in nature, consisting of plasma minus protein; and contains flakes of mucus, shed epithelial cells, and enormous numbers of vibrios. With continued loss of water and bicarbonate, marked dehydration and metabolic acidosis become prominent features, and the affected person is in a state of collapse. This is referred to as the second stage of the disease, characterized by circulatory failure, subnormal temperature, and anuria. In contrast to bacillary dysentery, the mucous membrane of the bowel remains intact.64, 131 Uncommonly, the infection may not result in diarrhea but give rise to the form of the disease known as cholera sicca; an occurrence taken as evidence of a generalized intoxi-

Treatment is symptomatic and consists

of the replacement of fluid and electrolytes. Usually there is acidosis, and patients literally minutes away from death may be saved by rapid (as much as 1 liter in 10 minutes) intravenous administration of isotonic bicarbonate solution. Thereafter the patient is rehydrated with alkali, bicarbonate or lactate, or saline, and is maintained on intravenous fluid to balance the loss until the diarrhea stops and renal function is initiated. Usually 20 to 25 liters are required, but in exceptional instances as much as 70 liters have been required. Potassium loss may be sufficient to result in cardiac difficulties, and replacement of potassium as a constituent of the rehydration fluid or by mouth as green cocoanut water is more often required in pediatric than adult cholera.92

Antibiotic therapy of cholera has, in general, been without effect other than a reduction in the time during which vibrios are excreted. Tetracycline, however, markedly reduces fluid requirements. 71, 135 Other antibiotics and the sulfonamides, to which the vibrios are sensitive *in vitro*, show little or no such effect; the mechanisms involved are not as yet known.

The case fatality rate in untreated cases is 50 to 60 per cent, although it may be less in some epidemics, ¹⁰⁴ perhaps 10 to 20 per cent in treated persons, and may be reduced to 1 per cent or less under ideal conditions of therapy.

Carriers. On recovery the vibrios disappear rapidly and are discharged for only a short time. In the series of 200 cases studied by Ying, 98 per cent were negative by the end of the second week, only a few giving positive cultures as late as the third and fourth weeks. In a study of 113 continuously observed cases, Gilmour⁶⁹ found that 71.6 per cent were negative after the first week, 89.3 per cent after two weeks, and 98.1 per cent after three weeks, with maximal periods of intermittent excretion of 20. 21, 23, and 25 days. Similar results were observed in the Egyptian epidemic of 1947, with 96 per cent of patients negative after three weeks and with a maximal period of intermittent excretion of 39 days.

It is probable that causal carriers occur during epidemics, and such a carrier state was demonstrated by Egyptian workers during the 1947 epidemic. A carrier incidence of 2 to 4 per cent in contacts was observed, but Gohar and Makkawi found

that it did not persist more than 10 days. More recently, similar results have been obtained in the Philippines and adjacent areas and in Bengal. In the Calcutta carrier study, it has been found that the infection persists in asymptomatic carriers during interepidemic periods, when only occasional cases occur, and in East Pakistan, where one study¹¹² showed that in 49 infected households the infections were nonclinical in 17. In the Philippines, asymptomatic infections or ambulatory cases functional as carriers have been observed repeatedly since infection was reintroduced in 1961.

For many years it was believed that no chronic carrier state occurred in cholera, a conclusion based in part on the failure to find such carriers by bacteriological examination and in part on epidemiological evidence such as the failure of cholera to occur in Cevlon in spite of the large annual influx of migratory workers from India, where cholera persists in endemic form. 118 With the spread of infection with biotypes other than the "classic" vibrio, e.g., El Tor types, it has become clear that a chronic carrier state may occur. One carefully studied individual in the Philippines has continued sporadically to excrete the vibrio biotype with which she was originally infected for a period of several years, but how widespread such a chronic carrier state may be is as yet uncertain. The method of purging to detect the carrier state may be useful in this connection.63

Bacteriological diagnosis.^{12, 101} As indicated above, the vibrios are present in the rice water stools in very large numbers and can usually be isolated from fresh specimens without difficulty. At times they are present in sufficient numbers that they can be found in stained smears, preferably made from a flake of mucus. The fluorescent antibody technique has been applied to the rapid identification of the vibrios.⁴⁶ Direct darkfield examination of the rice water stool specimen with and without antiserum permits rapid provisional diagnosis as immobilization of the cholera vibrios in the presence of specific antiserum.³

Both enrichment and direct streaking of agar mediums are used for cultures. For enrichment a few drops of the stool are added to a tube of alkaline (pH 8.0 to 9.0) peptone water. The vibrios grow much more rapidly than the other intestinal bacteria, and after six to eight hours' incubation they

form a thin film of growth on the surface of the medium which can be smeared and stained, and streaked on agar.

A number of agar mediums have been used for isolation of the cholera vibrio, including starch-phenolphthalein agar and the alkaline blood medium of Dieudonné. Bile salt agar, nutrient (meat extract) agar at pH 8.0 and containing 0.5 per cent sodium taurocholate,34 is one of the most useful and satisfactory mediums for primary isolation; some workers believe that the medium is improved by the addition of gelatin. On 24 hours' incubation the cholera vibrios grow as small to moderate size translucent smooth colonies sharply differentiable from the roughened colonies of coliform bacilli. Oblique light microscopy is considered by many to facilitate recognition of the typical colony.87 The microorganisms are identified by slide agglutination in O group I antiserum and typed in monospecific Inaba and Ogawa antiserums if desired. With clinically typical cholera in an epidemic this is sufficient identification.

The isolate may also be typed with monospecific Inaga and Ogawa antiserums, the Heiberg type being shown by fermentation tests, and the biotype determined by application of the various differential criteria described above. Tests other than O group I agglutination are usually carried out at a central or reference laboratory.

Epidemiology.^{40, 80} As in the other enteric infections, the connecting link in the dissemination of cholera is between infected feces and the mouths of susceptible persons. In consequence, the disease is frequently waterborne and may be transmitted by any food ordinarily consumed in the raw state. The quantitative importance of contact infection is not known. Cholera differs from the other enteric diseases, however, in the highly explosive character of the outbreaks, which is attributable to the short incubation period, the high case fatality rate, and its rapid disappearance when the outbreak has subsided.

In a sense cholera is one of the easiest to control of the highly contagious diseases, for it cannot spread when sanitary facilities, i.e., sewage disposal, water supply, etc., are in efficient operation. A striking instance of this was reported in the Balkan War in 1913 in which infection was widespread in the Bulgarian Army around Sofia, but cases in the capital were largely imported and the disease showed little tendency to spread

there; Sofia was efficiently sewered and had an excellent water supply.

Endemic foci. Cholera persists in interepidemic periods in foci of endemic infection. Endemic areas are adjacent to rivers and are low lying and densely populated. In such areas tank water and other stored water often contain cholera and related vibrios.1,2 It is probable that the infection persists in contact, and possibly chronic, carriers, and as a small number of human cases of the disease, which occur continuously between epidemics. 17, 26 The use of phage typing in tracing sources of infection has not as yet been too productive, for there is considerable overlap in sensitivity, and only a few phage strains have been used. 105 It is not known why this occurs in some areas but not in others.

The classic focus of endemic infection is in Bengal, extending from the Ganges and Brahmaputra deltas into Assam and Bihar. There is also a focus of infection in Burma in the Irrawaddy delta, and possibly also in parts of the Salween delta. Cholera has occurred in Nepal for many years, and this area is also suspected of being an endemic focus. The disease occurs in China, but precise information as to its prevalence and possible endemicity is lacking. Following the spread of the so-called El Tor biotypes, infection has persisted in endemic form in a number of invaded areas in southeast

Asia and in the Philippines, but it is too early to say whether such areas have become permanent foci of infection.

Epidemic spread. Each year the disease occurs in epidemic form, first in the endemic areas such as Bengal, beginning in February. The peak is reached in April and May in Bengal, just before the beginning of the monsoon season, and there is a remarkable correlation between relative humidity and the incidence of cholera. 124 In Bengal a small secondary peak occurs in December; the seasonal incidence of the disease in that area is illustrated in the accompanying figure.

The disease extends from these foci each year, reaching a peak in Punjab, in July and August, and in Uttar Pradesh in the spring, and spreads through central India to Madras and Bombay.

Its spread beyond these areas into Europe and the Western Hemisphere has occurred as a series of pandemics, as described at the beginning of this chapter. The quarantine stations at Tor and Basra function to prevent the spread of infection into the Middle East, although cholera occurred in epidemic form in Egypt in 1947, coincident with the British evacuation of India. The traditional route of spread to the west has been via Afghanistan into the Middle East and Eastern Europe, and this spread is difficult to control because of the continuous

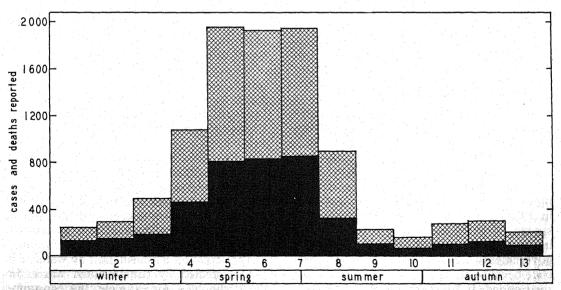


Figure 122. Representative incidence of cholera in West Bengal, shown as the mean numbers of cases and deaths reported by four-week periods. (Data from Epidemiological and Vital Statistics Reports, World Health Organization.)

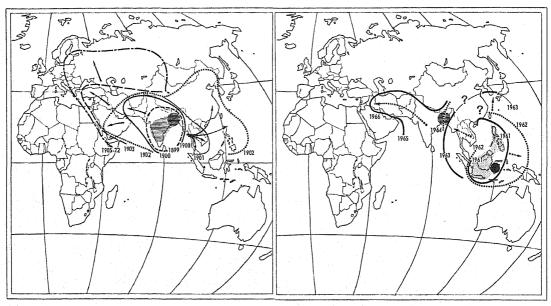


Figure 123. The epidemic spread of cholera. Left, the classic route of spread through Afghanistan and the Near East to Europe and Africa as illustrated in the sixth pandemic, 1899–1922. Right, the spread of cholera during the period 1961–1966, which appears to approximate the initial stages of a pandemic. (World Health Organization.)

operation of smuggling and similar unregulated traffic. Although cholera has not occurred in Western Europe since the latter part of the nineteenth century, and not in European Russia since 1926, an outbreak occurred in the Ukraine in 1943.⁵⁶

El Tor cholera.37 The occurrence of cholera caused by biotypes other than the "classic" cholera vibrio in Indonesia in the late 1930's has been noted above. The infection spread from the initial focus of infection in Celebes to other parts of Indonesia, and to Sarawak and possibly Kwantung, and was also found in India as early as 1945.110 Beginning in 1960-61 it developed into epidemic form, appearing in Macao and Hong Kong,91 probably from Kwantung, progressed to the Philippines,30,136 spreading through the western Pacific area to Borneo. Loas, Thailand, Cambodia, etc., and into India. 108 Further spread has followed the traditional route into the Middle East to produce epidemics in Iran in 1964 and Iraq in 1965. It is difficult to ascertain the true spread of the disease, since it comes under international quarantine regulations, and governments are reluctant to admit its presence because of the imposition of quarantine restrictions. It has been suggested that extension of the disease is characteristic of the beginning of a world-wide pandemic,

and it has been a cause of considerable concern.

Because of quarantine restrictions, what continues to be popularly called El Tor cholera was considered to be "choleriform enteritis" until this terminology became untenable, and international quarantine regulations were modified in 1962 to eliminate the difference in control measures between cholera and El Tor cholera.

The initial differentiation of the El Tor biotypes was based on hemolysin formation,42 but with the persistence of infection in the invaded areas, the hemolytic characteristic has tended to disappear, with the appearance of "nonhemolytic El Tor" in Indonesia, 103 and current isolates in the Philippines tend to be nonhemolytic; insensitivity to phage type IV appears to be a more stable character. The disease produced is clinically indistinguishable from classic cholera, the treatment is the same, and vaccines prepared from the "classic" biotype show complete cross-protection against El Tor challenge under experimental conditions.116

The infection is transmitted by water and foods infected by contaminated water. In the Philippines, for example, the consumption of contaminated shrimp apparently was responsible for the initial epidemic.

On invasion of an uninfected area, the disease tends to occur in all age groups, but with persistence of the disease it is found more often in children, and pediatric cholera tends to characterize the disease in endemic areas. It tends also to be a contact infection under these circumstances, although waterborne and foodborne outbreaks occur as in Hong Kong four years after the initial epidemic.⁵⁰

Immunity. Recovery from an attack of cholera confers an immunity to subsequent infection, but the quality of the immunity is not definitely known; it appears to be of limited duration, perhaps six months to a year. There is a rise in serum antibody. agglutinin, vibriocidal antibody,44 and antitoxin. According to the most recent studies, agglutinin reaches a peak of perhaps 1:1280 and vibriocidal antibody 1:50,000 to 1:70,000 10 to 12 days postonset,128 and antitoxin about 2000 units per ml. at two weeks.82 Coproantibody is produced also. but may not parallel serum antibody.55 It is not clear how such serum antibody titers may relate to an effective immunity to the disease but, under appropriate conditions. they may have diagnostic value. It may be noted, though, that cross-reaction with Brucella antigens may result in cholera vibrio agglutinin titers in brucellosis serums, and that very high-and inexplicablecholera vibriocidal antibody titers are occasionally observed in serums from persons having no contact with cholera vibrios.

Vaccines. The vaccines currently used for prophylactic immunization consist of saline suspensions of killed vibrios, not less than 8000 million per ml., and are usually bivalent, consisting of equal numbers of the Inaba and Ogawa serotypes. One vaccine is put up in freeze-dried form, and that prepared at the Haffkine Institute is a whole culture vaccine of vibrios grown in casein hydrolysate medium. Immunogenic potency is assayed by the active mouse protection test; i.e., immunized mice are challenged with mucin-vibrios by the intraperitoneal route (see below). 117 Immunization of man is commonly accomplished by a course of two doses of 1 ml. each, given a week apart by the subcutaneous route, and the booster inoculation, required at sixmonth intervals by international regulation, is 1 ml. subcutaneously or 0.1 ml. intrader-

The validity of the mouse potency test is

questioned perennially, and field tests suggest some small degree of efficacy²⁹ which may or may not be correlated with the mouse test. The most recent of such field tests are those carried out in Calcutta under the auspices of the World Health Organization. in the Philippines, 121 and in East Pakistan by the Pakistan-SEATO Cholera Research Laboratory. 111 The vaccines tested varied widely, the most effective being the CRLtested vaccine, which gave about 75 per cent protection in an endemic area. Other immunizing preparations, notably purified lipopolysaccharide antigen,83,140 have been suggested, but as yet have not been adequately field-tested.

The pathogenesis of the disease, i.e., the production of symptoms, even though the vibrios remain in the lumen of the bowel and out of contact with circulating antibody. has been taken to suggest that coproantibody plays a significant part in immunity. Coproantibody response is best obtained by local application of antigen, e.g., by the oral route,⁵⁴ and under experimental conditions appears to be associated with an antibacterial immunity, 13 but oral antigen has not been field-tested in man. An attenuated living antigen would presumably be most effective, and such vaccines have been suggested. 107 The appearance of antitoxin in response to infection and the observation that asymptomatic contact carriers have high antitoxin but low antibacterial antibody titers (i.e., they tolerate the infection without symptoms) suggest that an antitoxic component may be a significant element in an effective immunity.

Pathogenicity for lower animals. The cholera vibrio appears to be exclusively a parasite of man, and naturally occurring infections, as distinct from contamination as of crustaceans, has not been described. Experimental infections have been of interest in connection with the assay of immunogenic potency of vaccines and as models simulating to a greater or lesser degree the human disease.

Experimental enteric infection of the guinea pig was described by Koch, who fed the animals broth cultures of the vibrios, as a part of the evidence for the etiological relation of the vibrio to the disease. The infection may be asymptomatic, but a fatal infection may be produced with streptomycin-resistant vibrios in animals treated with the antibiotic.⁵² The suckling rabbit

may be infected by the oral route, as shown by Metschnikoff at the turn of the century, and this infection was studied in detail by Sanarelli in the 1920's. The guinea pig may be infected by intraperitoneal inoculation, and a fulminating fatal bacteremia may be produced in the mouse by the intraperitoneal inoculation of vibrios in mucin. This last is used extensively in the assay of immunogenic potency of vaccines as indicated above. A fatal infection may be produced in the embryonated egg, 45, 65, 66 and the embryo is also killed by cell-free toxins of type 1.

In the 1950's, De and his co-workers showed that infection may be produced in ligated segments of the small bowel of the adult rabbit by direct inoculation,24 a technique which has been used with enteropathogenic coliforms and Shigella as described elsewhere (Chaps. Twenty and Twenty-two). Subsequently De showed that the ligated loop reaction can be produced with cell-free lysates of the vibrios or with liquid culture supernatants.22 At about the same time. Dutta and his associates restudied the infant rabbit infection, and showed that diarrhea, often fatal, could also be produced in these animals with similar cell-free preparations.33 Russian workers have made some use of the infection in the ligated ileal loop in the guinea pig. Quite recently Sack, Carpenter, and their colleagues have found that an enteric infection may be produced in the dog and that cell-free preparations give similar reactions: the resultant disease closely resembles that in man but the required doses are relatively large.

Animal models. Of these experimental infections, the enteric infections have been of greatest interest as models simulating to a greater or lesser extent the human disease, and the production of essentially identical reactions with cell-free material has shown the overriding importance of toxins. The infant rabbit model has been used extensively by Finkelstein and his co-workers in their study of the cholerigenic

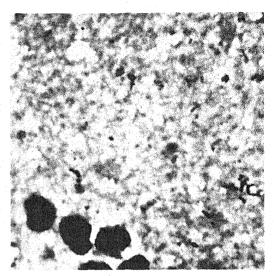


Figure 124. Vibrio cholerae in peritoneal exudate of a guinea pig. Note the swollen and aberrant forms. Gram stain; × 1250.

toxins described above, and the use of this model by others has made possible the differentiation of enterotoxic and sodium transport-inhibiting toxins. The infant rabbit model cannot be used for active immunization studies, but it has been applied in the assay of passive protection.⁹⁶

The reaction in the rabbit ileal loop model to cell-free toxin resembles the human disease in the histopathology produced,⁵¹ and in the toxin-induced water and ion movement indicated by the composition of the intraluminal fluid.90 It can be precisely quantitated to allow the titration of toxin and antitoxin,81 and the antitoxin titration can be modified as an interference reaction for the titration of toxoid.82 It has been found that the ileal loop reaction involves three separable toxins: that which induces fluid accumulation; type 1 toxin, which is absorbed in consequence of altered bowel permeability; and type 3 toxin, which inhibits active sodium transport. The antitoxin titrated is that to the first of these, which has been prepared in purified form. 18, 76

Other Vibrios

For the most part as an adjunct to the study of the cholera vibrio, a considerable number of vibrios have been isolated from water and from the feces of individuals

suffering from mild diarrheal disease, socalled cholera nostra. Some of these have been given place names such as V. danubicus, V. ghinda, and V. massauah. A phosphorescent vibrio, *V. phosphorescens*, has been isolated from water, and *V. proteus* from human feces. All of these differ immunologically from the cholera vibrio, *i.e.*,

belong to O groups other than I.

A number of other vibrios are known which produce disease in animals but which are apparently not pathogenic for man. Vibrio metchnikovii was isolated in 1888 from fowls suffering from an epidemic disease resembling fowl cholera. It closely resembles the cholera vibrio morphologically and physiologically and is highly pathogenic for guinea pigs and pigeons, while the cholera vibrio is not pathogenic for the latter. It differs from V. cholerae immunologically and is neither agglutinated nor lysed by anticholera serum.

An epidemic disease of carp and other fishes caused by a vibrio designated V. piscium has been described by David. The vibrio resembles the cholera vibrio morphologically and is immunologically re-

lated to it.

Vibrio parahaemolyticus. 142 A form of foodborne infection prevalent in the summer in Japan, and associated with the consumption of uncooked fish and shellfish (shirasu), has been found to be attributable to a halophilic vibrio, V. parahaemolyticus. In peptone water culture the vibrios form protoplasts, and they grow well in 7 per cent sodium chloride, but not in 10 per cent solutions. They are separated into two groups, 1 and 2, on a physiological basis and contain a number of differentiable O antigens. The vibrios of group 1 appear to be pathogenic, while those of group 2 are not. They are found in sea foods, in as many as 13 per cent of the samples in the summer months, and in healthy human subjects, as well as in some 30 per cent of cases of acute gastroenteritis. These microorganisms are quite unrelated to the cholera vibrios.

Vibrio fetus. A microorganism designated Vibrio fetus infects cattle, sheep, and goats to produce abortion in pregnant females and an essentially symptomless infection in males. The vibrio differs sharply from the cholera and paracholera vibrios in that it requires enriched mediums for growth, is relatively inactive biochemically, 88 and is antigenically distinct, falling into a number of serotypes. The naturally occurring disease is closely reproduced in the pregnant female guinea pig. The infection is transmitted by contact, and in cattle 8, 75 and

goats, but apparently not in sheep,⁷⁹ the infected male acts as a carrier, spreading the infection by breeding. In the female, agglutinating antibody occurs in the cervicovaginal secretions to considerable titer and is considered to be diagnostically reliable in contrast to irregular serum agglutinin. A few human infections have been reported,^{84, 130} some of which resemble brucellosis; the infection is a generalized one and the vibrios are found in the blood.

Two other vibrio species are responsible for diarrheal disease of certain domestic animals. *Vibrio jejuni* infects calves; it differs from *V. fetus* culturally and serologically and is nonpathogenic for guinea pigs. *Vibrio coli'* is the etiological agent of swine dysentery.³¹

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Chapter Twenty-four

BRUCELLA

Undulant Fever; Contagious Abortion of Cattle

In 1887 Bruce, while investigating the human disease known as Malta fever, Mediterranean fever, or undulant fever. discovered a microorganism in the spleen in fatal cases of the disease which he designated Micrococcus melitensis. A disease of goats transmissible to man, this affection not only is common on the island of Malta, where British garrisons have been often seriously affected, but occurs also on neighboring islands and on the shores of the Mediterranean Sea, and has been occasionally reported from India, South Africa, the Philippines, and the West Indies. It was first brought to attention in the United States about 1911.

In 1897 Bang, in Denmark, isolated a microorganism responsible for contagious abortion in cattle, an affection now commonly known as Bang's disease, which he termed *Bacillus abortus*. The isolation and cultivation of this bacterium in the United States were first recorded by MacNeal and Kerr in 1910.

These two diseases, one primarily of goats and secondarily of man, and the other, one of cattle, were long studied quite independently, and apparently no connection between the two was recognized prior to the work of Evans in 1918. She demonstrated the remarkably close morphological, cultural, and serological relationship existing between these bacteria, which are now recognized as being intimately related to one another.

In 1914 Traum isolated from fetuses prematurely expelled from sows a bacterium which is now known to be closely related to the bacterium of Bang's disease and that of undulant fever. Regarded as three species, these bacteria have been given the generic name of Brucella and are designated as Brucella melitensis, Br. abortus, and Br.

suis. Infection with these bacteria is termed brucellosis.³

Morphology and staining. The Brucella are small coccoid or short bacillary forms varying from 0.4 to 3.0 μ in length and from 0.4 to 0.8 μ in breadth. Some variability is noted, and both coccoid and bacillary forms may appear intermingled. There is a greater tendency to the coccobacillary form in Br. melitensis than in Br. suis, with Br. abortus intermediate between the two, but no distinction can be made on a morphological basis. The microorganisms usually occur singly or in pairs, and in cultures short chains may be found. The smooth forms are encapsulated but spores are not formed, and these bacteria are nonmotile.

On semisolid mediums the colonies are small, circular, convex, amorphous, smooth, glistening, and translucent. No pigment is formed, but the growth of *Br. melitensis* becomes brown in older cultures, and the browning extends down into the medium. This browning is shown by some strains of *Br. abortus* also.

Brucella may be stained by the usual aniline dyes, but there is a tendency toward irregular staining and, in some cases, bipolar staining. They are gram-negative.

Physiology. The nutritive requirements of these bacteria are relatively complex, and the best growth is obtained on enriched mediums such as liver infusion broth or agar. Brucella have been cultivated on amino acid synthetic mediums; some strains require nicotinamide, thiamin, and pantothenic acid while others require biotin also. A number of chemically defined or synthetic mediums have been prepared which support the growth of Brucella, one of the simplest of which is that of Gerhardt and Wilson¹⁹ containing lactate, glycerol, asparagin (or glutamic acid or histidine), thiamin, nicotinic

acid, pantothenic acid, and biotin together with inorganic salts. No growth occurs at 6°C. or 45°C., and the optimum temperature is 37°C. Neither acid nor gas is produced from carbohydrate mediums, although it may be shown that glucose is utilized to a small extent, and its inclusion generally favors growth. Nitrates are reduced, and growth in milk is accompanied only by slowly increasing alkalinity. Gelatin is not liquefied, and indol is not formed. Urea is hydrolized by *Br. suis*, but not by *Br. abortus* or *Br. melitensis*. The optimum pH is 6.6 to 6.8

Hydrogen sulfide. All three species produce hydrogen sulfide but differ in that Br. suis produces it in abundance, Br. abortus to a lesser extent, and Br. melitensis to only a slight degree. It may be noted that ammonia is produced to a greater extent by Br. melitensis than by the other two species.

Carbon dioxide. These bacteria are aerobic, and Br. melitensis and Br. suis may be grown on primary isolation under the usual aerobic conditions. Br. abortus, however, requires incubation in an atmosphere containing 10 per cent carbon dioxide on primary isolation. Subsequent transfers from the primary growth must be incubated in 10 per cent carbon dioxide, but after a number of transfers Br. abortus adapts itself to ordinary aerobic growth.

Dyes. The species of Brucella show a differential sensitivity to a number of dyes. Thionine and basic fuchsin may be incorporated in liver infusion agar in dilutions of 1:50,000 and 1:25,000 respectively. Br.

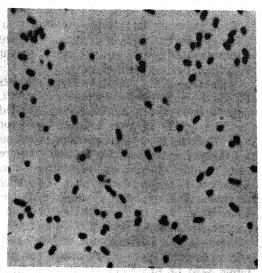


Figure 125. Brucella melitensis in pure culture. Note the coccobacillary morphology. Fuchsin; × 1050.

Differentiation of Brucella Species

DIFFERENTIAL REACTIONS	BR. MELI- TENSIS	BR. ABOR- TUS	BR. SUIS
Thionine 1:800	-	+	
Basic fuchsin 1:200	-	- -	. +
Basic fuchsin 1:200 Crystal violet 1:400	- -	_ * :	+
Pyronine 1:8000	· _	_	+
5 Inositol	_	+	_
.5 Maltose	-	tin n i in i	+
Mannose		+*	+
Rhamnose	- - '.	· · ·	_
Maltose Mannose Rhamnose Trehalose		<u> </u>	+
CO ₂ required	· · · · · · · · · · · · · · · · · · ·	+†	
Hydrolysis of urea	(slow)	(slow)	+ (rapid)
Formation of H ₂ S	±	+	++

^{*}Usually.

melitensis and Br. abortus will grow in the presence of basic fuchsin but Br. abortus will not, while Br. melitensis and Br. suis will grow in the presence of thionine but Br. abortus will not. Crystal violet and pyronine give the same reactions as basic fuchsin, and azure A and safranine O have some utility also. The growth of Br. melitensis is also inhibited in the presence of sodium diethyldithiocarbamate, while that of the other two species is not. Growth inhibition may be tested by the use of dve tablets analogous to the antibiotic discs used to assay antibiotic sensitivity, but higher concentrations are required than when the dye is incorporated in the culture medium; a zone of growth inhibition 4 mm. or more in diameter is taken to indicate sensitivity to the dye. The fermentation of a number of sugars, inositol, maltose, mannose, rhamnose, and trehalose, also has differential value. The physiological differentiation of Brucella species has been studied intensively8, 43, 44 and certain of the differential reactions are summarized in the accompanying table. Investigation of metabolic patterns among the Brucella species, such as the metabolism of amino acids, carbohydrates and Krebs cycle intermediates in respirometers, has shown a correlation of such patterns with species which sets

[†]On primary isolation.

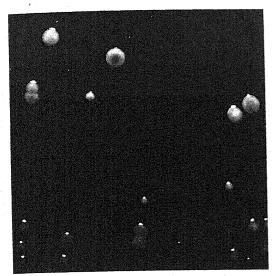


Figure 126. Colonial morphology of Brucella melitensis on liver infusion agar. The slight granularity is accentuated here, and the colonies are smooth, flattened, and slightly brownish in color. × 4.

Br. suis sharply apart-but also serves to characterize Br. melitensis and Br. abortus.36,37 A phage isolated by Russian workers is effective against Br. abortus but not the other two species.7, 10, 30 Phage sensitivity has been found, too, to correlate with oxidative metabolic patterns.35

The status of these physiologically differentiable types, reinforced by correlated differences in antigenic character (see below), as species of the genus Brucella, and their stability, appears to be established.63 The precise status is probably not of importance since Brucella species, like other species of microorganisms, are no more than form species, and the significant element is a generally accepted nomenclature.

The Brucella show the usual susceptibility to heat and disinfectants. A point of some practical importance is the rapid death of these bacteria at pasteurizing temperatures; both Br. abortus and Br. suis are killed in three minutes at 143° to 145° F. They persist in soil, water, and dust for one to two months but disappear within 10 days in milk, presumably in part as a consequence of the presence of acids formed by other bacteria. In this connection it is of interest that these bacteria are able to survive two hours or more in milk mixed with gastric juice.

Antigenic structure. Each of the three Brucella species contains two heat-stable antigens, designated as A and M. Br. melitensis contains a relatively large amount

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of M and a small amount of A, while both Br. abortus and Br. suis contain large amounts of A and small amounts of M. The ratio of A to M is said to be about 20:1 in the case of Br. abortus and 1:20 in the case of Br. melitensis. It is possible, then, to differentiate Br. melitensis from Br. abortus and Br. suis by serological methods, i.e., agglutination, but Br. abortus and Br. suis cannot be differentiated from one another in this way. Application of the gel-diffusion technique has shown a precipitating antigen in Br. melitensis which is seemingly not present in Br. abortus;46 This is perhaps the M antigen. In practice monospecific serums, i.e., serums adsorbed to remove the small amount of antibody common to the other immunological type, should be used. Immunoelectrophoretic analysis of sonic lysates of Br. abortus has shown that more antigens are present, at least

nine precipitable components.23

Toxin. No exotoxin is formed by these bacteria, but the cell substance of Brucella is toxic and the toxin has been found to be a component of the cell wall.14,31 A toxic antigen, prepared by tryptic digestion of dried cells followed by trichloracetic acid extraction and alcohol precipitation,42 appears to be a glucolipidpolypeptide complex. Further study, especially by Miles and his co-workers, has shown that the toxicity may be prepared as a highly purified material aminopolyhydroxy antigen, designated with a mouse LD₅₀ of 0.1 to 0.2 mg., and is hydrolyzed by dilute acid to phospholipid, formyl amine, and free amine, the last representing an amino sugar converted to a nonreducing anhydride during hydrolysis.38,39 The endotoxin appears to be substantially the same, in both nature and amount, in virulent and avirulent smooth strains. While it is considered probable that it contributes to the pathogenesis of the disease⁶⁰ and is thus a component of virulence, virulent strains appear to be able to multiply intracellularly, while the rough avirulent strains do not.6, 15, 16, 24 The nature of virulence is not yet clear. 49, 68

Variation. The Brucella species dissociate relatively easily to give rise to the rough form. The environmental factors affecting the dissociative process have been studied in detail by Braun. The $S \rightarrow R$ transformation is accompanied by a change to a rough colonial type, a loss of virulence, and alteration in immunological specificity. A point of particular interest is the relationship ingle-out-file and the part of the file for

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of alanine to the $S \rightarrow R$ dissociation; that produced by the S form accumulates in the culture to toxic concentrations, and the S form is replaced by the alanine-resistant R form. Other environmental factors, including oxygen tension, contribute also. ^{2, 55} The antigenic alteration begins to take place before morphological changes are apparent and has been a source of considerable difficulty in the serological typing of these bacteria; the altered immunological types have been termed paramelitensis or para-abortus strains. It is, therefore, essential to use only smooth cultures in the serological differentiation of these bacteria.

A number of tests have been devised for the detection of antigenic variants. The rough forms are, of course, spontaneously agglutinated in saline. Slightly rough strains may be detected by their agglutination upon boiling in saline for two hours (thermoagglutination test) or by slide agglutination with acriflavine. The smooth colonies may be differentiated morphologically by culture on glucose-glycerol agar for 96 hours, flooding the plate with 1:2000 aqueous crystal violet, and examining after 15 seconds by oblique transmitted light; the smooth colonies are a light blue-green, while the nonsmooth vary from red to blue-red or violetred.67

Pathogenicity for lower animals. 28, 50 Brucellosis is primarily a disease of domestic animals and is only secondarily communicated to man; the chief animal reservoirs are goats, cattle, and swine. It is of some interest that host specialization of the parasites has taken place, giving rise to the three species or, as some prefer to regard them, varieties of Brucella.

Goats.64 Goats may be artificially infected with Br. melitensis by almost any route, and it is probable that under natural conditions the vaginal discharge at the time of aborting and shortly thereafter plays an important part in the dissemination of the infection. Agglutinins appear in the blood of artificially inoculated pregnant goats by the third or fourth day, and the titer rises rapidly to perhaps 1:1000 within 48 hours and reaches a peak of 1:2000 or thereabouts by the twelfth day of infection. Just before the peak in agglutinin titer, bacteremia is initiated which persists for perhaps one month. This acute generalized infection becomes localized during the second month after the termination of the pregnancy during which the animal was infected. In most cases the bacteria do not persist in the udder and

uterus after the fifth month following termination of pregnancy. A second pregnancy does not, as a rule, cause an exacerbation of the disease, but in some cases the infection may remain localized in the area of the genital tract for several years.

The most obvious clinical symptom of infection is abortion, but it does not occur consistently. Pyrexia is apparent within 48 hours of the generalized infection, and there is slight diarrhea. The placenta is not retained, but a copious vaginal discharge is frequently observed for two or three weeks after kidding. In lactating goats the milk may be physically altered and appear in extreme cases as a clear fluid containing suspended clots.

Immature goats are highly resistant to the infection, and kids born of infected dams may not be infected and commonly do'not become so in spite of the ingestion of enormous numbers of Brucella in the milk. Nonpregnant mature goats are also resistant to infection and respond to artificial inoculation with only a low and transient agglutinin titer in the blood serum.

Brucellosis in sheep is similar to that in goats.

Cattle. Brucellosis in cattle is most commonly an infection with Br. abortus, although both Br. melitensis and Br. suis have also been found. The microorganism may gain entrance by a variety of routes, including direct inoculation into the vagina, by way of the conjuctiva, through the unbroken skin, or via the alimentary tract. The primary symptom of the disease is abortion of the fetus by pregnant cattle. The time elapsing between initial infection and abortion varies from three weeks to four months, and the period of gestation at which abortion may take place varies from two to nine months. Cattle do not abort, however, unless infected during pregnancy and even then not all abortperhaps 30 per cent-or the cattle may become sterile. Subsequent pregnancies may proceed normally in spite of persistence of the infection; second abortions are not common, and third abortions are rare.

The bacilli may be found in the blood in perhaps 10 per cent of the cases and are very likely consistently present during the acute infection. Early in the infection the bacteria are found in the lymph glands about the head and intestines, by the end of the first month they are found all through the body, and by the end of the third month they have localized in the mammary glands and

are found only in the udder. The invasion of the udder results in an acute or chronic inflammation with lesions in the alveoli and interalveolar connective tissue and, when the lymph glands are involved, a chronic lymphadenitis. Chronic infection of the udder may persist indefinitely without significant differences in the quality of the milk, and bacilli may be excreted over a long period, perhaps for life. The uterus, on the other hand, frees itself of the bacteria relatively soon, and the vaginal discharges do not contain the bacilli for an extended period.

Animals infected during pregnancy show an agglutinin titer ranging from 1:200 to 1:1000 which falls slowly over a period of six months or so. Cattle that continue to excrete bacilli in the milk generally show persistent agglutinin titers of 1:200 or more, although a titer of 1:50 has diagnostic significance. Agglutinins are also present in the milk and may be demonstrated in the whey after clotting with rennin. Infected animals become sensitized to the bacillary substance, and a skin reaction may be elicited by the intradermal injection of a preparation of Brucella protein designated as abortin or brucellergen. As in the case of the young goats, calves are relatively resistant to the infection.

Swine.29 Brucellosis of swine seems always to be due to infection with Br. suis. though these animals may be artificially infected with Br. abortus. In contrast with cattle, the males are commonly infected. and abortion in infected females is less frequent than in cows; about 50 per cent of swine abortions are due to unknown causes, not brucellosis. The clinical symptoms may be mild or lacking, and in a number of instances there has been no outward evidence of the disease in an infected herd, but the proportion of swine infected is as high as 20 per cent in some localities. Under natural conditions infection may take place via the alimentary tract. The infected boar, a testicular infection, is undoubtedly a significant element in the dissemination of the infection. The bacilli are eliminated with aborted fetuses and vaginal discharge, urine, semen, and milk.

Other animals. A number of other animals have been found to be naturally infected with Brucella. There is some evidence that the disease of horses known as fistula of the withers or poll evil is a Brucella infection; both Br. abortus and Br. suis have been isolated from cases of the disease.

Stone⁶⁴ found that 9.5 per cent of horses tested in New York City gave positive serological reactions. Brucella infections of fowl have been reported; in one instance Br. suis was isolated from several naturally infected birds, but the disease is probably not common. Dogs may also be infected naturally; Br. suis and Br. abortus have been isolated. Wild rats may be artificially infected, and Br. abortus has been isolated from a naturally infected rat. Natural infection of rabbits with Br. melitensis has also been reported. Of the usual laboratory animals, guinea pigs are readily infected and are most often used for experimental purposes. A fatal infection is produced in the mouse, using the mucin technique,9 and the embryonated egg is readily infected. 18, 32

Pathogenicity for man. 21, 41, 56 Man is susceptible to infection with these three species of Brucella, but infections with Br. melitensis and Br. suis are usually more severe than those with Br. abortus. The incubation period of undulant fever in man is highly variable and relatively long; it may range from one week to not less than four months. The case fatality is low-2 to 3 per cent. It may have varied clinical manifestations, and five types are recognized: (1) the intermittent type with shifting articular rheumatism, weakness, night sweats, and a temperature near normal in the morning but rising to 101° to 104° F. in the evening, in which the patient remains in bed in the latter part of the day; (2) the ambulatory type with much the same symptoms but to a mild degree; (3) the undulant type, generally melitensis infections, characterized by step-like increases in the temperature from day to day to a maximum and, after a time, gradual decrease in temperature and possibly successive repetitions of this sequence of events; (4) the malignant type, almost always melitensis infections, in which the temperature is high and sustained with extreme hyperpyrexia before death; and (5) an atypical chronic type which may take the form of muscular stiffness, gastric disturbances, and various neurological symptoms. In general, undulant fever is a disease of relatively long duration, one to four months, and relapses during convalescence are not infrequent. In a chronic form undulant fever may be difficult to diagnose; or it may be effectively subclinical as indicated by the isolation of Brucella from apparently healthy persons. A group of laboratory infections provided a unique opportunity for the study

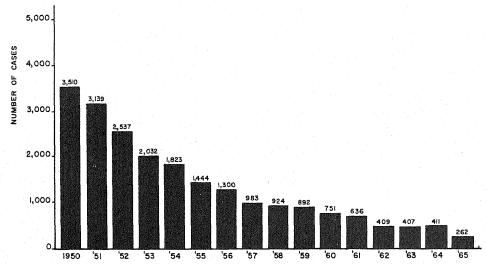


Figure 127. Cases of human brucellosis reported in the United States, 1950–1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

of the evolution of the disease in man;⁶⁵ an interesting feature was the association of neurosis and emotional disturbance with the prolonged convalescence.

Brucellosis in man is a generalized infection as a rule, while in lower animals it is a localized infection, particularly in cattle. The bacilli may be isolated from the blood stream in man,17 and the development of agglutinins is a diagnostic aid. Man also becomes sensitized to the cell substance of the bacteria, a hypersensitivity that is sometimes manifested as skin eruptions which may be macular or resemble the rose spots of typhoid fever. Localization may occur, however, and meningitis and meningo-encephalitis are probably not so rare as has been supposed, while in some instances orchitis, cholecystitis, endocarditis, and other local manifestations have been reported. Pulmonary lesions with infiltration of the hilar glands or lung tissue proper are occasionally observed. Pulmonary infection has led many investigators to suspect infection by inhalation, and Elberg and Henderson¹² have shown that the guinea pig may be experimentally infected by an intake of about 36 microorganisms inhaled as an aerosol. Brucella infection may be associated with abortion and mastitis in the human female in rare instances. As indicated above, the endotoxin appears to contribute in large part to the pathogenesis of the disease, both directly and indirectly as a hypersensitivity.57

Epidemiology. 22, 47 Brucellosis in is probably always acquired from infected domestic animals; man-to-man transmission is a possibility but rarely, if ever, occurs. The commonest modes of infection in the United States are the use of raw milk from infected cattle, and direct contact with the flesh of infected animals, both cattle and swine. As indicated above, animals may be readily infected via the alimentary tract, and it is not unreasonable to suppose that man is infected in this way also. The discharge of Br. abortus in the milk of infected cattle, then, provides the opportunity for infection when the milk is ingested in the raw state, and in many instances undulant fever is aguired in this way. Br. abortus has been found in certified milk in a number of localities. The pasteurization of milk, of course, provides adequate protection from this source of infection.

The penetration of the unbroken skin by Brucella has been pointed out earlier. Man may be infected by the handling of the tissues of diseased animals or by close contact with other infectious material; presumably the bacilli enter through minute abrasions in the skin or possibly through the intact skin. Employees of slaughterhouses, veterinarians, sausage-makers, and butchers are, of course, particularly exposed to infection by this means, and the incidence of brucellosis in this group is disproportionately high, with a tendency to be an occupational disease. It is probable that most infections with

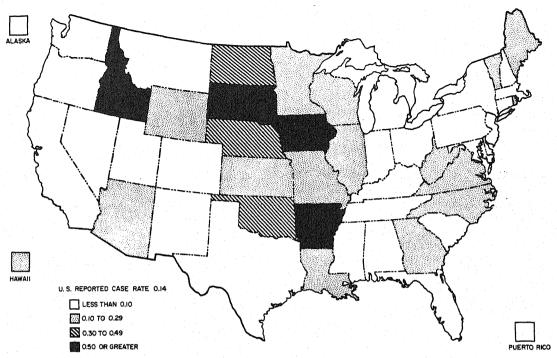


Figure 128. Geographical distribution of reported cases of human brucellosis per 100,000 in the United States in 1965. (Mordibity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Serivce, U. S. Department of Health, Education, and Welfare.)

Br. suis are aquired in this manner, although in some instances cattle are infected with this species and man aquires a suis infection via raw cow's milk. The epidemiology differs somewhat from one locality to another. In Iowa the incidence is highest among packing house workers and male farm workers, and in Indiana direct contact appears to be most important. In Utah, however, infected dairy products are the most important source of infection. In general, brucellosis is more common in males than females, in a ratio of about 4:1.

Laboratory infections with Brucella are very common, and even the most skilled workers have acquired undulant fever through working with these bacteria. Meyer and Eddie³⁴ reviewed 74 cases up to 1940, of which 44 occurred in competent bacteriologists. These infections are, in all probability, a consequence of handling infectious material and penetration of the skin by the microorganisms.

Undulant fever may also be acquired by drinking raw goat's milk, but infections with Br. melitensis from these animals are thought to be relatively infrequent in the United States. A number of cases have been found in North Carolina, Kansas, and Texas, and

it is known to occur in the Southwest in general where goat's milk is consumed. In Arizona the incidence of *Br. melitensis* infection is directly related to the goat population and has declined coincident with a reduction in the numbers of goats, while *Br. abortus* infection has remained high.

It has been shown experimentally that brucellosis may be transmitted by mosquitoes and biting flies, but at present there is no indication that this mode of transmission is of any significance in nature. Water is apparently not a vehicle of transmission; the single waterborne outbreak that has been reported was in the nature of a laboratory accident.⁴⁰

The prevalence of human brucellosis is not known with any degree of precision. The reported incidence of brucellosis reached a peak in 1947 with 6321 cases, declined to 3139 in 1951, 1300 in 1956, to 636 in 1961, and to 240 in 1966; the number of reported cases tends to reflect general interest, or laxity in reporting. The midwestern states, Iowa, Minnesota, and Wisconsin, contributed 25 per cent of all cases in the 10-year period 1942 to 1951 and 50 per cent of cases reported in 1951. The disease is found in rural areas for the most part, and the inci-

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554 **BRUCELLA**

dence in urban areas is very low. The incidence is high in the midwestern states and, in geographical distribution, tends to parallel the extent of the hog-raising industry. The incidence of brucellosis in cattle is relatively high, about 4 per cent of adult females, and more or less uniform throughout the country. Swine brucellosis is not so common; about 3 per cent of hogs show significant agglutinin titers. The estimated annual loss to the livestock industry is about 100 million dollars.

Bacteriological diagnosis. 26, 53 The laboratory diagnosis of brucellosis involves the demonstration of the causative microorganism and of specific antibodies. When the bacterium can be isolated and identified the diagnosis is solidly established, but the presence of antibodies indicates only an immune response to what may have been a past rather than present infection and is, therefore, only suggestive.

Brucella is more often found in the blood stream in man, particularly in the pyrexial period, but cannot always be isolated. Blood or blood clot is added to tryptose broth in 2 to 5 ml. amounts and incubated in an atmosphere containing 25 per cent CO₂. The enrichment culture should be subcultured at four-day intervals and, if subcultures are negative, carried for a period of not less than three weeks. For subculture, agar plates of liver infusion or tryptose agar should be inoculated. The bacteria may be identified by agglutination with antiserum; differentiation between Br. melitensis and Br. abortus-Br. suis may be made by agglutination with type-specimen antiserum, and the culture tested for H₂S production and growth in the presence of thionine and basic fuchsin.

A presumptive diagnosis made on clinical grounds may be partially, but by no means absolutely, confirmed by an agglutinin titer of 1:320 or higher or by a rising antibody titer during the course of the disease. Unfortunately, agglutinin titers differ from one laboratory to another, and there is pressing need for the use of a standard agglutinating antigen and agglutination procedure. 62 A rapid test has been devised by Ruiz Castaneda51 which does not require laboratory facilities.

Blocking antibody occurs which interferes in the direct agglutinin titration. There has been considerable interest in the diagnostic utility of incomplete, or blocking, antibody titration by the antiglobulin method.20,66,69 The significance of such titers

is somewhat uncertain; it is believed that incomplete antibody tends to be formed relatively late in the disease, and possibly may be taken to indicate the presence of active infection, e.g., continued antigenic stimulus.

Interpretation of the agglutinin response is also complicated in some instances in which the individual has received cholera vaccine, for Brucella and the cholera vibrio contain common antigens. The allergic response to Brucella cell substance, demonstrable as a skin reaction of the delayed type, is now regarded as quite unreliable for diagnostic purposes.

The agglutination test is widely used in the diagnosis of brucellosis in cattle. Agglutinins are present in milk as well as serum, and in the latter case a "ring test" (the Abortus Bang Ringprobe, or ABR test) is commonly used. It consists of mixing one drop of hematoxylin-stained bacteria with 1 ml. of milk, incubating for one hour at 37°C., and reading; the agglutinated bacteria are carried to the surface with fat globules to

form a colored ring.

Chemotherapy. 13, 52, 58 Chemotherapy of brucellosis has been disappointing, and the disease responds only irregularly and temporarily to administration of sulfonamides and the antibiotics. There is some evidence that combined therapy, as with sulfadiazine plus antibiotics or a combination of tetracyclines and streptomycin, may terminate acute clinical episodes, but relapses occur, ranging from 20 to 50 per cent. Of the antibiotics, the tetracyclines are considered to be the most effective.

Immunity.²⁷ The resistance of calves and nonpregnant cows to clinically apparent brucellosis is clearly an expression of natural immunity, though the older animals respond to the microorganism with the production of antibodies and the development of an increased resistance to subsequent infection. Man likewise appears to have a high degree of natural resistance to the infection, and it is probable that there are many more infections than clinical cases of brucellosis. In the series studied by Huddleson and Munger in which exposure to infection was known, only about half the individuals showing evidence of infection by an immune response had clinically apparent disease.

In man the immune response is evidenced by the appearance of agglutinins, opsonins, bactericidal antibody,4 and hypersensitivity to preparations (brucellergen) of the cell

substance of the bacteria. It is not clear. however, that this response is associated with an increased resistance, i.e., effective immunity, to the infection. The available evidence suggests than an effective immunity is primarily a cellular immunity. 25 Consistent with this, the therapeutic use of antiserums in human brucellosis has given disappointing results.

Vaccines. 48 The possibility of producing an effective prophylactic immunity, not only in man but also in animals, is of continued interest. Vaccines consisting of killed suspensions of the bacteria induce a serum antibody response, demonstrable as agglutinins. etc., but do not produce an immunity to the disease.

An avirulent variant of Br. abortus, strain BA 19, has been studied extensively as a possible immunizing agent. It differs from virulent strains of Br. abortus in that it does not require carbon dioxide, is relatively avirulent for the guinea pig, and is inhibited by thionine blue. Used in calves, it does not prevent infection but modifies the disease as indicated by a sharply reduced rate of abortion and shedding of the virulent bacteria. It is not completely avirulent for man, and a number of accidental infections have been reported.^{54, 61} In man it does not produce chronic disease, but induces a degree of hypersensitivity such that subsequent contact with Brucella antigen gives severe reactions.⁵⁹ It has been administered to man in Russia, reportedly to as many as 11 million persons, and is said to reduce the incidence of brucellosis as much as 10-fold.45

An avirulent mutant of Br. melitensis, designated Rev 1, has been studied by Elberg and his co-workers. It behaves in the guinea pig as if it were a virulent organism until the infection becomes established; it appears to have little pathogenicity as an intracellular parasite, and the lesions disappear,33 Similar results have been obtained with monkeys, using only a few hundred cells as inoculum, to produce an immunity effective against subsequent challenge with virulent bacilli.11 It appears highly promising, but as yet is in an experimental stage.

BRUCELLA BRONCHISEPTICA

This microorganism is very similar to Br. abortus but is motile and highly aerobic. It does not produce hydrogen sulfide. It is

immunologically related to Br. melitensis and Br. abortus but can be separated from them by agglutinin-absorption tests. It also resembles Hemophilus pertussis both culturally and immunologically. Originally isolated from dogs ill with distemper, it is not now generally believed to stand in any causal relation to that disease. It is, however, frequently found as the cause of bronchopneumonia in guinea pigs and other rodents.

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PASTEURELLA AND ACTINOBACILLUS

Hemorrhagic Septicemia; Plague; Tularemia; Glanders; Actinobacillosis

Pasteurella

The group of bacteria making up the genus Pasteurella consists of biochemically inactive, nonmotile, gram-negative bacilli which often show bipolar staining. The group is relatively homogeneous, with the exception of the tularemia bacillus, which is set apart by its fastidious nutritive requirements and certain other characteristics, and is differentiable by physiological properties as shown in the accompanying table. These bacteria are primarily pathogens of lower animals.¹¹ The etiological agent of fowl cholera was among the first of them to be described, and was the bacterium used by Pasteur in his early studies on immunity.

Pasteurella multocida (Pasteurella septica). The group of animal diseases loosely designated as hemorrhagic septicemias, and characterized by large and small hemorrhagic areas found in the subcutaneous tissues, serous membranes, muscles, lymph glands, and throughout the internal organs, are considered to be caused by a single species, Past. multocida or Past. septica. Strains may differ widely in virulence and to a certain degree in host specificity, and for many years were given corresponding names, viz., Past. aviseptica or Past. avicida, the cause of fowl cholera and pathogenic for mammals as well; Past. muriseptica or Past. muricida, pathogenic for rodents but not fowl; Past. leptiseptica or Past. cuniculicida, associated with contagious nasal catarrh, or "snuffles," of rabbits; Past. suiseptica or Past. suilla, the bacillus of swine

plague which is pathogenic for both mammals and birds; and *Past. boviseptica* or *Past. bollingeri*, found in domestic animals.

These bacteria may be grown on the conventional nutrient agar mediums, and show no hemolysis on blood agar although the colonies may become darkened. They occur as distinct colonial types, smooth, mucoid and rough. The smooth forms are iridescent when examined by oblique transmitted light, and Elberg and Ho37 described three types, the green or golden being the most virulent, and the red and blue of decreasing virulence; the noniridescent mucoid type is avirulent. They are separable into four groups on the basis of arabinose-xylosedulcitol fermentations which approximate, but do not necessarily coincide with, immunological type; characterization as types is immunological rather than biochemical. Roberts differentiated four serotypes, designated I, II, III, and IV, and this was carried further by Carter,20 who designated the types B, A, C, and D corresponding respectively to Roberts' types. The immunological specificity is attributable to specific polysaccharide, and is demonstrable by precipitin, capsular swelling, and passive hemagglutination reactions; effective immunity is type-specific. A lipopolysaccharide has been isolated109 which is both immunogenic and toxic. There is an association between colonial morphology and antigenic characteristics;21 specificity is a property of the smooth form, and mucoid strains are often not typable.

Differentiation of Pasteurella Species

SPECIES	motility (grown at 18°–22° C ₆)	GROWTH* ON MCCONKEY'S AGAR	INDOLE	H ₂ S	MALTOSE FERMEN- TATION†	
Past. septica Past. hemolytica Past. pseudotuberculosis Past. pestis	+	<u> </u>	+	+ + + +	++	

^{*}Sparse, disappearing after two to three days' incubation.

†Acid, no gas.

The pathogenicity of Past. multocida is sometimes considered to be open to question because of the relatively common occurrence of the bacteria in normal animals. It appears, however, that strains isolated at random are of widely varying virulence. and those found in normal animals are commonly the avirulent mucoid form. Virulence appears to be associated with serotype to the extent that type B (I), which is largely confined to Southeast Asia, is the etiological agent of classic hemorrhagic septicemia occurring in cattle and other domestic animals. The other types are associated with fowl cholera and bovine and swine pneumonias in this country and in Europe.

Human infections are reported from time to time⁴⁷ and, curiously, have been found more often in Oregon in recent years than elsewhere in the world.⁵⁴ The A and D

serotypes tend to predominate, suggesting domestic animals as sources of human infection.²²

Pasteurella hemolytica. This bacterium is closely similar to *Past. multocida*, but is differentiable by its formation of indole and failure to ferment maltose, and is immunologically distinct. It appears to be the cause, or a common cause, of shipping fever of cattle,²³ but is apparently able to produce disease under natural conditions only when the resistance of the animals is reduced by stress. It is also a cause of ovine mastitis, and has been called *Past. mastitidis*.

PASTEURELLA PESTIS — THE PLAGUE BACILLUS^{44, 51, 78, 103, 104}

Plague is an ancient disease, originating in central Asia and/or central Africa. Whether the Biblical plague of the Philistines in 1320 B.C. was plague of Pasteurella etiology as contended by some, or an epidemic of dysentery and piles (the last is difficult to believe) as asserted by others, is uncertain, but the great pandemic during the reign of the Emperor Justinian in 542 A.D. was certainly plague.

Plague prevailed extensively throughout Europe during the Middle Ages. It has been estimated that 25,000,000 persons, or one-quarter of all inhabitants of Europe, perished in the "great mortality" or "Black Death" of the fourteenth century (1348-49). Few diseases have left so deep a mark on general literature. Boccaccio's *Decameron* contains one of the most vivid descriptions of the plague ever written, and Defoe's

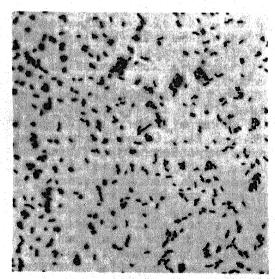


Figure 129. Pasteurella multocida. Smear from a pure culture. Fuchsin; × 1050.

Cres I Turk grafic

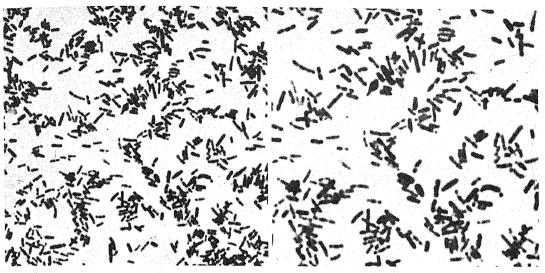


Figure 130. The plague bacillus. Smear from pure culture; fixed in methyl alcohol and stained with methylene blue to show bipolar staining. Note the involution forms present even at 24 hours' incubation. Left, \times 1050; right, \times 2100.

fictitious* Journal of the Plague Year provides a realistic picture of the devastation of London in 1665 by an outbreak of Black Death in which 70,000 persons perished.

For reasons that may be only partly conjectured, plague has had irregular periods of quiescence and recrudescence. Western Europe has been practically free from the plague since the middle of the eighteenth century, and the disease began its first great extension in modern times with its appearance in 1893 in Hongkong and in 1896 in Bombay. Plague caused great loss of life in British India; official statistics show that in the period from 1896 to 1918 more than 10,000,000 deaths were due to this disease. In October, 1899, a case was recorded at Santos, Brazil; this is thought to be the first occurrence of the plague in the Western Hemisphere. Plague first appeared in the United States in San Francisco in 1900: it is assumed that it was introduced by infected rats from the Orient. The infection apparently spread to ground squirrels and other wild rodents in the western part of the country.

Plague continues to decline, but this is considered to represent a continuation of its decline from its most recent epidemic prevalence, rather than a prelude to its extinction. In fact, the infection is now more firmly established in endemic foci than at

any time in the past, and it may be a disease of the future as well as of the past.³

The plague bacillus, *Past. pestis*, was discovered almost simultaneously by Yersin and by Kitasato in 1894.

Morphology and staining. The plague bacillus is a short, plump, ovoid rod 0.3 to 1.25 μ in length. In the body fluids the bacillis may occur in pairs, but long chains are rare, and, in general, there is no characteristic arrangement. The bacilli are nonmotile and a surface slime layer, polypeptide in nature, is present. The presence of this material, as an envelope or capsule, is associated with resistance of the bacilli to phagocytosis.⁵⁹ Involution forms are common, especially in older cultures, and coccus shapes, large rods, and gigantic swollen forms may be observed. The tendency of the plague bacillus to aberrant morphology is accentuated by cultivation on mediums containing 3 to 4 per cent sodium chloride; the appearance of involution forms in 24hour cultures on salt-containing mediums has been regarded by some as a characteristic of differential value.

Colonies on nutrient agar or gelatin have a delicate, drop-like appearance, with a round, granular center and a thin, granular, uneven margin. On blood or other hemincontaining mediums the colonies are dark brown in color, the pigmentation being derived from absorption of hemin from the substrate.⁵⁷

The plague bacillus is uniformly gram-

^{*}Defoe was only four years old in the year of the great plague.

negative and shows a marked tendency toward polar staining, i.e., there are heavily stained areas at the ends of the cell separated by a lightly stained area in the center. For good bipolar staining the smear should be air-dried and fixed in alcohol. The usual aniline dyes, such as methylene blue, are satisfactory. The plague bacillus is best demonstrated in tissue sections by a polychrome stain.

Physiology. Past. pestis is not nutritionally fastidious, and growth occurs on all the ordinary culture mediums, although in pentone water it is very poor. Unselected strains require amino acids as sources of nitrogen, and certain amino acids appear to be essential for some strains;50, 112 apparently bacterial vitamins are not required. Casein hydrolysate has been widely used as a base for liquid mediums in the production of vaccine at the Haffkine Institute¹¹⁸ for many years, and more recently in studies of toxin and other antigenic products, but hematin, blood, or possibly reducing agents are required for growth on the surface of a solid medium. Unlike the case with most of the bacteria pathogenic for man, a temperature of 25° to 30° C. is more favorable than one of 37° C., and the limiting temperatures for growth are -2° C. and 45° C. In any case, the colonies on solid mediums grow slowly and never attain a large size. The plague bacillus is aerobic and facultatively anaerobic.

Sugar fermentations are variable, including that of glycerol, and a small amount of acid but no gas is produced. Neither coagulated serum nor gelatin is liquefied, and indol is not produced. Nitrates may be reduced to nitrites, and a small amount of hydrogen sulfide is formed. On potato and in milk multiplication is slow and scanty; milk is rendered slightly acid but is not curdled.

Devignat has distinguished three physiological varieties of the plague bacillus. 31, 102 Variety I, Past. pestis orientalis, does not ferment glycerol but reduces nitrate; variety II, Past. pestis antiqua, ferments glycerol and reduces nitrate; and variety III, Past. pestis mediavalis, ferments glycerol and does not reduce nitrate. The last ferments melibiose while the first two do not, to provide an additional differential character. 93

The primary foci of infection of variety I are in India, Burma, and south China;

variety I was the agent of oriental plague which caused the 1894 epidemic and it is also responsible for sylvatic or wild rodent plague in the western United States. Variety II, probably the oldest, came from Transbaikalia, Mongolia, and Manchuria in central Asia, moved west with the Aryan invasions, and followed the valley of the Nile into central Africa, leaving foci that persist to the present time. It also moved back toward the Mediterranean in the sixth century and is believed to have been responsible for the Justinian plague that spread through the Roman Empire. It has since disappeared from Europe and has remained isolated in Africa. Variety III, possibly arising by a slow transformation of variety II, spread from the Caspian Sea throughout the whole of Europe, causing the Black Death, and established itself in endemic foci.

The plague bacillus does not exhibit any marked resistance to deleterious influences. Exposure to drying, particularly at the higher summer temperatures, kills it within a short time. The bacillus is quite sensitive to the action of sunlight and chemical disinfectants; it is killed, for example, by 0.5 per cent phenol in 10 to 15 minutes and by heating to 55° C. in about the same time. Cultures kept in the refrigerator, however, remain viable over long periods. In general, *Past. pestis* does not survive long outside the animal body and disappears rapidly from soil, water, and buried cadavers.

Toxin. The toxicity of the plague bacillus appears to be intermediate between endotoxin and exotoxin. The toxicity is not associated with somatic glucolipid-poly-

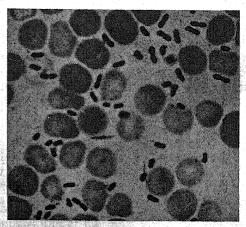


Figure 131. The plague bacillus in the blood of an infected mouse, \times 2000. (Douglas and Wheeler.)

peptide complexes as in the case of the enteric bacilli, but, while it appears to be protein in nature, it does not diffuse freely into the surrounding medium like the diphtheria, tetanus, and botulinus toxins. Toxic extracts have been prepared by freezing and thawing of suspensions, followed by centrifugation and filtration, and by autolysis and separation of the toxic supernatant. Toxicity in soluble form may also be prepared by phage or bile salt lysis of the bacilli.

On local inoculation the toxin produces edema, often followed by necrosis, and the general effect appears to be largely on the peripheral vascular system and liver to produce shock which is irreversible when a lethal dose is given. It appears to have no selective action on the heart or central nervous system. The pathology in toxin-sensitive animals—mice and rats—is closely similar to that produced in infection, but this similarity is not observed in other animals.¹¹⁴

This murine toxicity has been prepared in highly purified, antigenically homogeneous form by salting out procedures and electrophoretic fractionation of extracts of acetone-dried bacilli.1 This material contains 18 amino acids, but no polysaccharide or phosphorus, has a molecular weight of about 74,000, and a mouse LD₅₀ of 0.1 μ g. or less. It splits NAD, inhibiting NADdependent reactions such as the oxidation of keto acids² and inhibits the respiration of rat and mouse heart mitochondria, but not that of mitochondria from toxin-resistant animals such as the rabbit.62 The murine toxin has been separated by electrophoresis into two toxic protein fractions differentiable from one another.⁹⁴ There is evidence²⁷ which suggests that the murine toxins do not account for all the toxicity of the plague bacillus.

Virulence.17, 120 The murine toxin is found in both virulent and avirulent strains of the plague bacillus, and interest in matters such as variation and antigenic structure has centered largely around the question of virulence. The usual S-R dissociation occurs, but the colonial morphology, and associated properties, is not related to virulence since both S and R forms may be either virulent or avirulent.36 Similarly, the plague bacillus is antigenically homogeneous in that serotypes, in the conventional sense, do not occur, but the antigenic structure is complex as shown by sensitive techniques such as gel-diffusion.25 The murine toxin is one antigenic component, and the polysaccharide-protein envelope substance. or antigen, designated Fraction 1, is another which is associated with virulence, but either or both may be present in virulent strains.

More recently the various factors associated with virulence have been clarified. The presence of Fraction 1 is associated with the resistance of the bacilli of phagocytosis, but doubt as to its indispensability to virulence is raised by the occurrence of virulent strains lacking, or containing only very small amounts of, Fraction 1; such strains have been isolated in the laboratory as mutants and in the naturally occurring fatal infection in man.¹²⁴

Two antigens, designated V and W and demonstrable by gel-diffusion, have been described, 18.75 which appear to be invariably

The Known Virulence Determinants in Pasteurella pestis*

	PROPERTY			LD ₅₀		
PURINE INDEPENDENCE†	PIGMENT ON HEMIN AGAR‡	CAPSULAR ANTIGEN	VW ANTIGEN	CA ⁺⁺ DEPENDENCE	MOUSE	GUINEA PIG
i kanan aya + masalah da	+	+	+	+	<10	<10
	**	+	+ +	+	>10 ⁸	>109
+		4	-1 √ 1 + -1 1 1	+	>108	>109
+	4		4	+	<10	~104
+	44	+			>108	>109
· · · · · · · · · · · · · · · · · · ·	+	4	+		>108	>109

^{*}Prepared by Dr. R. R. Brubaker.

[†]Strains lacking only the ability to synthesize purines are fully virulent when purine is inoculated simultaneously with the challenge dose.

[‡]Strains lacking only the ability to form pigment are fully virulent when Fe⁺⁺ is inoculated simultaneously with the challenge dose.

associated with virulence; they do not occur separately, but may vary in relative amount, and are thought to relate to the resistance of the bacilli to phagocytosis.

Certain nutritional requirements have also been associated with virulence, or avirulence, one is a temperature-dependent requirement for calcium⁵⁰ of virulent strains, and the others are nutritional deficiencies occurring in avirulent mutants. Of the latter, one relates to pigmentation of colonies on hemin-containing mediums noted earlier; nonpigmented mutants are avirulent unless the animal is inoculated concurrently with hemin or ferrous iron.58 There appears to be a relation between ferrous iron and pesticin I, a bactericin-like substance produced by wild-type strains and associated with coagulase and fibrinolytic activity, in the virulence complex.13 The other is a purine deficiency mutant for which virulence is similarly demonstrable when the deficiency is supplied at the time of inoculation.16 Such restoration of virulence to deficient mutants is presumably a matter of supplying nutritive requirements not satisfied by the tissues of the host, i.e., an effect on bacterial growth in vivo. Maintenance of virulence in culture is markedly influenced by environmental factors.97

In summary, a virulent strain of Past.

pestis may be defined as a toxigenic, encapsulated strain containing the VW antigen complex, forming pigmented colonies on hemin-containing mediums, and able to survive and grow in macrophages.²⁴

Pathogenicity for man. Plague in man appears most commonly in two forms, the bubonic or glandular plague and plague pneumonia. In the bubonic type the symptom complex is characteristic, and diagnosis on clinical grounds is relatively simple. From the buboes, which may be either primary or secondary, bacilli may pass over into the blood; in fatal cases the bacteria often multiply in the blood extensively. The case fatality is 60 to 90 per cent. Primary plague septicemia can also probably occur. There are sometimes subcutaneous hemorrhages. During the plague epidemics in the Middle Ages such hemorrhages seem to have been more frequent than at present, and the dark spots to which they give rise were the origin of the popular name. "Black Death."

Plague pneumonia⁸⁹ occurs secondary to the glandular infection and may be transmitted to give rise to primary plague pneumonia. Pneumonic plague is usually fatal. In this variety the sputum may contain enormous numbers of plague bacilli, and the infection is spread from man to man by

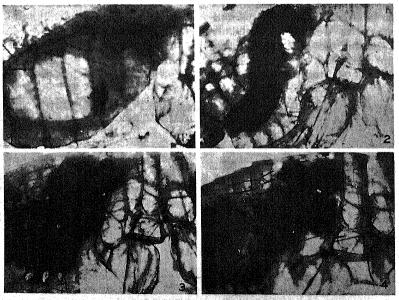


Figure 132. Plague bacilli in the infected flea; sections stained with methylene blue. The bacilli appear as dark stained masses. I, flea on the ninth day after infection. 2, eighteenth day after infection; note the stomach and proventriculus packed with bacilli. 3, twenty-second day after infection; note the large mass of bacilli and the swollen proventriculus. 4, twenty-third day after infection; the proventriculus is further enlarged. (Douglas and Wheeler.)

droplets. Because of this direct spread, pneumonic plague is by far the more dangerous type. The extensive outbreak of pneumonic plague in Manchuria in 1910 to 1912 is said to have caused approximately 60,000 deaths, the case fatality being practically 100 per cent. Both bubonic and pneumonic plague have been produced experimentally, giving in the monkey diseases which closely resemble the human infection. ^{53, 107, 119}

EPIDEMIOLOGY OF PLAGUE

A primary infection of the skin with the plague bacillus sometimes occurs (cutaneous plague) but does not seem to be common. Cases of mild plague, the so-called pestis minor, are met with in some epidemics, but it is probable that healthy persons do not carry plague bacilli for long, if at all. The occurrence of intestinal plague in man has never been clearly established.

Epidemiology. Plague is a disease of rodents which also occurs in man as sporadic cases and in epidemic form. The susceptible rodents fall into two groups: the domestic rodents, or rats, and various wild rodents. Of the former, the black house and ship rat, Rattus rattus, and the less susceptible gray sewer rat, R. norvegicus, are most commonly involved; the Egyptian rat, R. alexandrinus, may be infected also. Although both the feces and urine of infected rats may contain plague bacilli, and rats may be infected by feeding upon rats dead of the disease, the infection is most often transmitted by fleas. The Indian rat flea, Xenopsylla cheopsis, is a common, and perhaps the most effective, vector, but the rat flea of North America and Europe, Ceratophyllus fasciatus, may also transmit the disease. Plague bacilli are present in the blood during the acute disease, as many as 100 million per ml., and the flea is infected by feeding on the diseased rat.

When the plague bacilli are taken into the stomach of the flea they multiply, but do not spread to other parts of the body, and on microscopic examination large masses of bacilli may be seen in the infected flea. In some the bacilli multiply so rapidly that the bacterial mass mechanically obstructs the proventriculus so that little or no food may pass. In the effort to feed, plague bacilli are mixed with the drawn blood of the host, and regurgitated into the bite. The bacilli are also discharged in the feces, and infection may occur by contamination of the bite wound with fecal pellets.

A wide variety of wild rodents can be, and are, infected with plague, and this rodent disease, improperly referred to as sylvatic plague, constitutes the reservoir of infection. In the United States wild rodent plague was first observed in California in 1908, and has spread slowly eastward, reaching into Texas, Arizona, New Mexico, Oklahoma, and Kansas. Rodents such as prairie dogs, ground squirrels (Citellus), wood rats (Neotoma), and mice (both Microtus and Peromyscus) may be infected, 38, 65, 77 and there is evidence that rabbits may be involved in some areas.64 Various rodents are infected in other parts of the world:110 the sisel, Citellus pygmaeus, in the northwest Caspian area; various species of Meriones in Kurdistan; the Indian gerbil, Tatera indica, in India; the field rat, R. exulans, in Java; etc.

It is well known that plague exists in two kinds of endemic centers, temporary and permanent, and the status of the domestic rat as a reservoir of infection has been doubtful. Similarly, a postulated role of the rat flea in the dissemination of human epidemic plague has been subject to serious exception with the occurrence of epidemic plague in the absence of rats and their fleas. More recently the epidemiology of the disease has been considerably clarified. Extensive studies, beginning in Kurdistan, have made it clear (a) that the permanent reservoirs of infection are in relatively resistant wild rodents; (b) that foci of wild rodent plague tend to die out when the animals are susceptible to the disease; (c) that the domestic rat is no more than the "liaison rodent" between the wild rodent and man; and (d) that while infection is initially carried to man by ectoparasites of rodents, epidemic bubonic plague in man is transmitted largely from man to man by human ectoparasites, notably the human flea, Pulex irritans.

The epidemiology of plague falls into a pattern on the basis of the foregoing. For example, in Kurdistan four species of wild rodents constitute the reservoir of infection: Meriones libycus and M. persicus are highly resistant to plague, and M. tristrami and M. vinogradovi are extremely susceptible. The persistence of plague is a consequence of the balance among these species, and the sedentary habits of the resistant species whose burrows act as reservoirs of infected fleas. On occasion a human infection is acquired from this source, and this gives

rise to human plague in villages, which is transmitted by the human flea.8 When the villages are small and scattered, and communications slow, the epidemic is limited and dies out. Throughout this permanent epidemic area, including southeastern Russia, the infection persists in very many small permanent foci of infection where the various Meriones species are closely intermingled. In Java the field rat is resistant, and plague persists in permanent foci; the domestic rat is relatively rare in rural areas, too restricted in range to spread the infection and too susceptible to maintain it for any length of time.⁵ Consequently, plague in Java is typically sporadic, brief, and limited to villages. In the United States Microtus and Peromyscus are resistant to plague and for that reason are probably a more important reservoir of infection than the more susceptible ground squirrel.

In contrast, the focus of infection in Mesopotamia remains temporary, occurring in susceptible rather than resistant wild rodents, and tends to die out unless periodically reactivated by importation of infection.⁶ Similarly, in India the gerbil is somewhat resistant to plague infection, but not sufficiently so as to allow the development of permanent pockets of the disease, and plague in India appears to be epidemiologically unstable and will presumably die out unless infection continues to be reintroduced.^{4, 106} Introduction of infection into a healthy area can occur only where *R. rattus* is present, the epizootic is brief unless the more resistant *R. novegicus* is present, and these domestic rats serve to infect the wild rodent population.

The prominent role of the human body louse in epidemic plague in man points up the significance of disinfestation as a control measure, together with chemoprophylaxis, in epidemics. In the longer view, control of the rat population will prevent introduction of new infection and reduce the probability of human infections initiating epidemic spread. Control of wild rodent plague is a difficult matter, but encouraging results have been reported from Russia.³⁹

Bacteriological diagnosis.⁷ In man the bacilli are found in material aspirated from

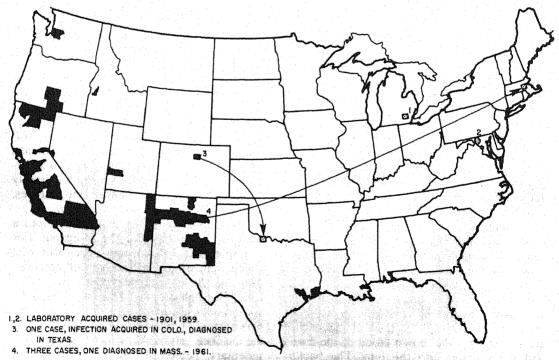


Figure 133. The occurrence of human plague in the United States as indicated by counties reporting one or more cases in the period 1900-1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

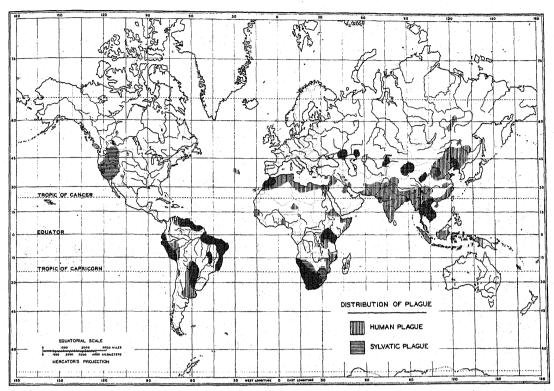


Figure 134. The world-wide distribution of human and sylvatic plagues. (Redrawn from map prepared by Army Medical Intelligence. Based on Goode Base Map No. 201M. By permission of the University of Chicago Press.)

buboes, in cultures or smears of internal organs, especially the spleen, and, in pneumonic plague, in the sputum. The presence of gram-negative, bipolar-staining, ovoid bacilli is highly suggestive. Blood cultures, taken late in the disease, should be cultured first in broth. Other material may be inoculated directly on blood agar and glycerol agar. An enriched azide-antibiotic-dye selective medium has been developed for isolation of the bacilli from highly contaminated material such as soil.72 Cultures may be identified by cultural and biochemical characteristics and by agglutination in plague antiserum. The bacilli show some tendency to spontaneous agglutination, and the slide agglutination test is unsatisfactory. Postmortem diagnosis may be made by tapping the liver, lungs, and buboes, if present, with a syringe. The serous fluid is used for preparing smears and, after dilution in saline, for the inoculation of guinea pigs. Microscopic examination of smears often gives almost unequivocal diagnosis, and fluorescent antibody staining may be useful when specimens are too grossly contaminated for culture or animal inoculation. 56 Animal inoculation should always be carried out when possible.

Guinea pigs may be inoculated subcutaneously or, with specimens that have undergone gross contamination and decomposition. by rubbing the material on the freshly shaved abdomen; the plague bacilli penetrate the minute abrasions whereas the contaminants do not. The animals die in two to five days; postmortem findings are characteristic and include subcutaneous and general congestion, congested spleen, granular liver, and pleural effusion. The bacilli may be found in spleen smears and elsewhere and cultured. It is important that the animal be freed of ectoparasites before inoculation. Plague in rodents may be diagnosed by postmortem findings, which are similar to those in the guinea pig, by microscopic and cultural demonstration of the bacilli and by guinea pig inoculation. Of the serological reactions, passive hemagglutination appears to be the most sensitive. 74, 90

Chemotherapy. 83, 86 Streptomycin and sulfadiazine appear to be the most effective chemotherapeutic agents in plague. Prophylactic inoculation with sulfadiazine or

sulfamerazine is reasonably effective for the protection of contacts. For therapeutic purposes, streptomycin is given for the first four to five days and then replaced by sulfonamide. In severe septicemic and/or pneumonic plague, the streptomycin may be supplemented with oral chloramphenicol or tetracyclines and antiserum prepared in the rabbit.

Immunity. 87, 88 Recovery from plague confers a solid immunity to subsequent infection. In this immunity phagocytosis appears to be of primary importance, functioning through opsonization to counteract the antiphagocytic properties of the envelope or capsule of the bacillus. It is probable that antibody to the envelope antigen, Fraction 1, plays a significant part in an effective immunity, and that to the VW antigen complex may be significant also. Antitoxic immunity does not contribute materially to effective immunity, and antitoxic serums are not protective.

While virulent strains are considered to be preferable for the preparation of killed vaccines, vaccines prepared with avirulent strains may also give an immunity as do certain living vaccines of avirulent strains. Various kinds of soluble preparations^{26, 66, 117} produce an immunity under experimental conditions, as indicated by protection tests in monkeys,³³ mice,¹⁴ or guinea pigs, but the immunity may not be of a high order. Differences in the response of animal species make for some difficulty in assaying immunogenic potency, *e.g.*, a preparation producing an immunity in mice may not immunize guinea pigs.

Immunization of man has been carried out with vaccines made up of suspensions of killed or attenuated bacilli or with whole culture antigen. The vaccine used by the United States Army during World War II consisted of a suspension of 2000 million formalin-killed virulent plague bacilli per milliliter, and was given in two doses, 0.5 ml. and 1.0 ml., seven to 10 days apart. The Haffkine Institute vaccine is a whole culture antigen. The plague bacilli are grown in nonantigenic casein hydrolysate medium decolorized with charcoal and containing Tween 80 to facilitate diffuse growth. It is standardized by immunogenic potency rather than by the number of bacilli con-

Inoculation with living attenuated bacilli

produces a solid immunity in experimental animals. The use of such vaccines in man has been investigated in South Africa with encouraging results, and a similar vaccine has been used in Indonesia in more than 10 million inoculations without untoward results. These vaccines have been of particular interest in Russia also.⁷³

PASTEURELLA PSEUDOTUBERCULOSIS⁷⁰

Past. pseudotuberculosis (B. pseudotuberculosis rodentium) resembles the plague bacillus closely, 121 but causes a disease of rodents, particularly guinea pigs. It is differentiated from the plague bacillus by its motility at 18°-22° C. (by stab culture in soft agar), its production of urease, and its fermentation of rhamnose and glycerol. 32, 43 It has been reported to grow abundantly as large opaque colonies on desoxycholatecitrate agar, while *Past. pestis* grows only scantily as reddish pinpoint colonies. 122 It is less virulent for the guinea pig than the plague bacillus, and produces a toxin, which differs from that of *Past*, pestis antigenically and in that rabbits and guinea pigs as well as rats and mice are susceptible to it.115 Past. pseudotuberculosis is separable into five serotypes and six subtypes. Certain of the somatic antigens are related to Salmonella antigens, and types II and IV are agglutinated by serum from persons infected with Salmonella. Diagnostic agglutination reactions must, therefore, be interpreted with caution, and serums should be absorbed with Salmonella antigen. Many of the antigenic components are shared with Past. pestis, including the VW complex.19

The natural mode of infection is probably by way of the alimentary tract. Subcutaneous inoculation of guinea pigs proves fatal in two to three weeks, with caseous swellings and nodules ("pseudotubercles," which have unfortunately given this bacterium its name) in various organs. Past. pseudotuberculosis has been found, though rarely, in animals other than the guinea pig. Human infection is rare and has been considered to be almost uniformly fatal; but less serious disease, commonly of lymphoid tissue and sometimes simulating acute appendicitis when the adenitis is mesenteric, is observed from time to time. 30, 52, 69, 116

PASTEURELLA TULARENSIS⁴¹

Tularemia is a disease of rodents, rabbits in particular, that is transmitted to man either directly through the handling of the flesh of infected animals or indirectly through an insect vector. Past. tularensis (Bacterium tularense) was discovered by McCov and Chapin in 1912 in a plague-like disease of the California ground squirrel. Tularemia in man, however, is contracted largely from the rabbit, and it was shown by Francis in 1919 that the disease known in Utah as deer fly fever is, in fact, tularemia transmitted from infected rabbits to man by the bite of the fly Chrysops discalis. Francis also found that Past. tularensis was present in rabbits sold in the markets of Washington. D.C., and that a disease known as rabbit fever was not infrequent among those in contact with the rabbits. Human cases have been observed throughout the continental United States.

Morphology and staining.48 In culture Past. tularensis is a minute, gram-negative pleomorphic rod 0.2 μ in breadth and 0.3 to 0.7 μ in length; the coccoid form predominates in young cultures and the bacillary form in older cultures. In smears from the spleens of infected mice or guinea pigs, the bacteria appear as coccoid forms in well-defined clusters. Capsules are present in the body: spores are not formed, and the microorganisms are nonmotile. The organism reproduces by a number of methods, including binary fission, budding, filament formation, and the like, and is regarded as closely related to the microorganisms of the pleuropneumonia group (Chap. Twentyseven). It is thought by some to be more closely related to Brucella than to Pasteurella and is, in fact, named Brucella tularensis by British workers; it has also been suggested that it be set apart and a new genus, Francisella, created for it.101

On solid mediums *Past. tularensis* forms minute, transparent, drop-like colonies that are mucoid in consistency and readily emulsifiable. Colonial variants, including those associated with virulence, are more sharply characterized when observed by oblique transmitted light.^{34, 35}

This bacterium is somewhat difficult to stain; methylene blue is not satisfactory, but either carbol-fuchsin or aniline gentian violet may be used. Bipolar staining may be observed.

Physiology. Past. tularensis differs sharply from the other members of this genus in that it will not grow on the ordinary mediums. It may be cultured on a coagulated egg-yolk medium or on blood dextrose cystine agar. Growth occurs in casein or gelatin hydrolysate liquid medium supplemented with biotin, blood cell extract, and liver extract, and a medium of heart infusion, dextrose, cystine, and hemoglobin will support good growth. Peptic digest of hemoglobin has been used to give a medium that is transparent rather than opaque. 42 It has also been cultivated in chemically defined mediums,81,125 spermine is required, and the pH is critical and should not be higher than 7.5. It is aerobic and facultatively anaerobic, and its optimum temperature is 37° C. Glucose, maltose, and mannose are fermented, the fermentation of glycerol, levulose, and dextrin is irregular, and mannitol, galactose, xylose, trehalose, salicin, arabinose, adonite, sucrose, lactose, amygdalin, dulcitol, erythritol, inositol, inulin, raffinose, sorbitol, and rhamnose are not fermented. Differential fermentations are of no value. It is killed by exposure to 56° C. for 10 minutes. It has been reported that this bacillus contains an endotoxin.

Pathogenicity. Two clinical types of tularemia are recognized, one the glandular or ulceroglandular type, which is the more common, the other the so-called typhoidal type. In the first instance the acute stage of the disease is characterized by headache, pains, and fever, and a papule appears,

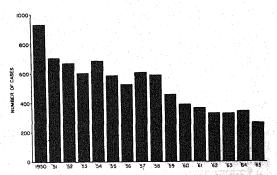


Figure 135. Cases of tularemia reported in the United States, 1950-1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

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frequently on a finger where presumably the bacilli enter the body, which later breaks down and forms an ulcer. The axillary and epitrochlear glands become painful and swollen and may break down with the discharge of purulent material. In persons infected via the conjuctiva, ulcers form on the inner surfaces of the evelids, and the cervical and preauricular glands may become tender and somewhat swollen. In the typhoidal type of the disease there are no local symptoms.

During the first week of illness the bacilli may be present in the blood and have been cultured, although, in general, cultures made directly from man are not successful, and the bacillus is best isolated by guinea pig inoculation and culture from necrotic foci found in liver, spleen, and lungs of the pig on autopsy. Direct cultivation from the blood is rarely possible but has been accomplished, and it has been suggested that during the first week of the disease initial bacteremia may occur which, in fulminating cases, develops into septicemia. The bacilli can only rarely be found in smear preparations from human cases. In the experimental disease the bacilli are present in the lymph spaces and phagocytic cells of infected tissues, and their presence within the cells in enormous numbers has caused some workers to suggest an intracellular proliferation of the microorganisms similar to that of the rickettsiae. Agglutinins are present in the blood in the second week of the disease and may persist in diminishing amounts for at least as long as 18 years after recovery. The average duration of the disease is two

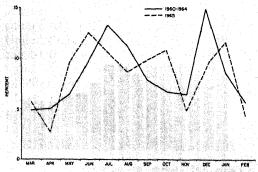


Figure 136. The seasonal incidence of tularemia as per cent of cases by the month in the United States. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

to four weeks. The case fatality is low, 4.8 per cent, and the pathology of the disease in man is not well known.82

A variety of lower animals have been found to be naturally infected—in addition to the ground squirrels and rabbits noted above, wild rats and mice, woodchucks, opossums, beavers, coyotes, deer, red foxes, ground hogs, muskrats, hogs, skunks, dogs, cats, and lambs. In the western United States sheep infection, with a mortality as high as 10 per cent, serves as a source of human infection. 60 The infection also occurs naturally in some birds such as sage hens. grouse, and quail.

Of experimental animals, the mouse, guinea pig, and rabbit are susceptible in that order. There appear to be two geographical varieties of *Past*, tularensis with respect to rabbit virulence, the American variety, which is the more virulent, and a European-Asiatic variety, which is less virulent; the former ferments glycerol and the latter does not.98 The LD50 of a highly virulent strain is 10 or less for any of these animals. but as virulence declines it is evident first in the rabbit, then the guinea pig, and finally the mouse.10

Epidemiology. As indicated above. tularemia is acquired by man from lower animals either directly or indirectly. The bacilli may enter the unbroken skin of the guinea pig, and possibly this may occur in man through the dressing of infected rabbits and other animals, or the microorganisms may enter by means of minute abrasions on the skin of the hand. The bacteria may penetrate the intact skin; in the mouse the LD₅₀ through the skin is two to five times that by mouth, and about 10⁷ times the parenteral dose.105 Eve infection occurs, and in fact, such infections were the first human infections with the bacillus observed. Over 90 per cent of the human cases in this country are contracted from rabbits, and it is estimated that about 1 per cent of wild rabbits are infected. Jellison and Parker⁶¹ have reported that the cottontail rabbit, species of Sylvilagus and S. floridanus in particular, is by far the most important source of infection in this country, accounting for more than 70 per cent of all human cases in North America. Many wild animals are naturally infected, and Burroughs et al. 15 have compiled a list of 48 naturally infected vertebrates. Laboratory infection is not uncommon; 56 cases acquired from dissecTULAREMIA 569

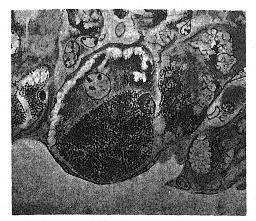


Figure 137. Pasteurella tularensis in hepatic cells of mouse. (Francis.)

tion were reported to 1940. Past. tularensis has also been found in streams and is perhaps associated with the epizootics occasionally observed in beavers. Present evidence indicates that fish cannot be infected with Past. tularensis and they probably play no part in the infection of water. Waterborne epidemics have occurred in Russia and Turkey. In this country infection of natural waters is relatively common in the northwestern states, and naturally occurring infections in beavers and muskrats are well known. In water and mud, infection may persist for many months, and available evidence suggests that the microorganisms may multiply there. 100 The infection is also transmitted by the ingestion of infected tissue¹²³ and this may be a factor too in the maintenance of the reservoir of infection in carnivorous animals.

The transmission of tularemia by an insect vector is common. In addition to the deer fly, C. discalis, Dermacentor andersoni, D. variabilis, D. occidentalis, Haemaphysalis leporis palustris, H. cinnabarina, and Ixodes ricinus californicus may carry it. In all probability the wood ticks serve to disseminate the infection in the animal population, and it is of particular interest that the infection is transmitted from the adult tick to the egg, and both the larvae and the nymphs are infectious. Tularemia may, then, be in part maintained in the insect population. In Arkansas, for example, the disease is mainly tickborne and is an occupational hazard among farm workers.

Tularemia has been found in all parts of the United States, in Japan, where it is also known as yato-byo or O'Hara's disease and in Central Europe, and extensive epidemics have occurred in Russia. Since World War II the infection has spread from southeastern Russia and Scandinavia west to the Atlantic and south to Asia Minor, and is now found in most of Europe.⁷¹

Bacteriological diagnosis. As indicated above, Past. tularensis is difficult to cultivate from infected material. Specimens should be streaked on blood-dextrosecystine agar. Characteristic minute, droplike colonies may appear in three to five days, but a culture should not be recorded as negative in less than three weeks. The procedure of choice is the intraperitoneal inoculation of a guinea pig with a saline emulsion of the specimen; relatively large amounts are required, usually 4 to 8 ml., of a fairly heavy emulsion. The animal should die in five to 10 days. The pathology is characteristic and includes hemorrhagic edema without pus at the site of inoculation, enlargement of the cervical, axillary, and inguinal lymphatics which contain dry caseous material, and small white necrotic areas in the liver and spleen. Smears and cultures may be made, but not infrequently the bacilli cannot be found or cultivated. In such instances the diagnosis depends on the guinea pig pathology.

Isolated cultures may be identified by specific agglutination. Conversely, patient's serum may be tested for agglutinins; a titer of 1:80 or higher is usually regarded as diagnostic if there are no Brucella agglutinins. In the event that Brucella is also agglutinated, the agglutination of *Past. tularensis* occurs more rapidly and to higher titer in tularemia.

Chemotherapy. The sulfonamides are almost completely ineffective chemotherapeutic agents in tularemia. Of the antibiotics, streptomycin has been found highly effective, subject to the rapid acquiring of drug resistance by the microorganisms; the tetracyclines are effective, but chloramphenicol has only slight activity.

Immunity. An attack of tularemia confers an effective immunity, but it is not as solid as once thought. Two extremely well documented cases of reinfection among laboratory workers have been reported by Green and Eigelsbach. Both had been immunized with vaccine; one subsequently acquired two clinical infections within three years, and the other, two infections within six years. Similar experiences have been

reported by others. 85 The relation of antibody response to effective immunity is not clear; in any case agglutinin appears not to be closely related to protective antibody. Antibodies (agglutinins) to *Past. tularensis* show some cross-reaction with *Br. meliten*sis and *Br. abortus*. Prophylactic inoculation with vaccines is not successful in experimental animals in that it does not protect against the injection of virulent strains. There is some evidence, however, that vaccine prophylaxis in man confers a useful degree of protection. 63, 113

Actinobacillus

The microorganisms grouped within the genus Actinobacillus are, like Mycobacterium (tubercle and related bacilli) and Corynebacterium (diphtheria and diphtheroid bacilli), more closely related to the fungi than many other bacteria. Nevertheless, they are classified with the Eubacteriales under the tribe Brucelleae, together with Brucella, Pasteurella, and Hemophilus, The distinctions between these last three are not sharp, and some of these bacteria are put in one genus by some workers, and in another by other workers. The microorganisms of the genus Actinobacillus tend to be set somewhat apart and include the causative agents of glanders and melioidosis, together with forms found in association with actinomycetes in certain kinds of actinomycosis (Chap. Thirty-two), producing disease which closely resembles certain of the fungous infections, known as actinobacillosis.

THE GLANDERS BACILLUS (ACTINOBACILLUS MALLEI)

Glanders is a disease seen, as a rule, only in the solipeds (horse, mule, ass) but is occasionally transmitted to other domestic animals, to wild animals, and to man. Early regarded by many as a spontaneous, non-infectious disease, the transmissibility of glanders was demonstrated in 1837 by Rayer, who infected a horse by inoculating it with material from a case of glanders in a human subject. The causative bacillus was discovered in 1882 by Löffler and Schütz, whose work was confirmed and extended by Kitt, Weichselbaum, and others.

Morphology and staining. The glanders bacillus is a small rod, straight or slightly curved, usually with rounded ends, and often of irregular contour. Rather wide variations

in size are observed; the average length may be taken as 2 to 5 μ and the average breadth 0.5 to 1 μ . In culture the bacillitend to be shorter and more uniform in size than those observed in pus smears. In pus they are sometimes found within the leucocytes but more often occur free. There is no special arrangement in such smears, but in culture the bacilli may occur in pairs and, in older cultures, produce filaments with swollen ends in which true branching may be observed. The bacilli are nonmotile and nonencapsulated, and do not form spores.

Colonies on agar are small, round, convex and amorphous in consistency. They are translucent and yellowish in color and upon aging (eight to 10 days) become more opaque, and the center may become light brown. The growth on potato usually exhibits a characteristic appearance; clear, amber,

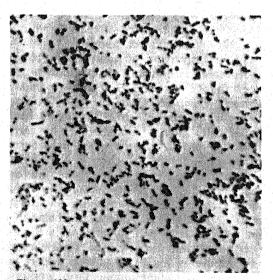


Figure 138. Actinobacillus mallei in pure culture. The generally poor staining is apparent, and bipolar staining may be observed in some of the cells. Methylene blue; × 1250.

honey-like colonies appear and may coalesce, and frequently the potato around the growth becomes tinged a greenish yellow, not unlike the discoloration produced by *Pseudomonas pyocyanea*. On horse blood agar the colonies are grayish green with browning of the medium but no hemolysis.

The glanders bacillus stains with the ordinary aqueous aniline dyes, though not readily. Best results are obtained with stains containing alkali, or a mordant such as phenol (Löffler's alkaline methylene blue. Ziehl's carbol-fuchsin). The bacilli are not acid-fast and are gram-negative. Cells from young cultures take the stain fairly uniformly, but those in older cultures stain irregularly, with a tendency to bipolar staining. Granules and coccus-like bodies within the cell take the stain somewhat more readily, and the bacilli have a beaded appearance in stained preparations. This irregular staining is due to the presence of lipid granules which do not stain with the usual dyes but may be demonstrated with Sudan black B or iodine-fuchsin.

Physiology. Growth occurs on ordinary nutrient mediums but is poor and slow on primary isolation. Forty-eight hours' incubation is generally necessary for the appearance on solid mediums of colonies 0.5 to 1 mm. in diameter. Growth is materially enhanced by the presence of glycerol, but glucose is without effect. A slightly acid reaction is favorable, and the optimum temperature is 37° C., though growth may occur over the range of 22° to 44° C. Growth on enriched mediums such as Löffler's serum medium or horse-blood agar is not markedly better than on glycerol agar. 91

The glanders bacillus is quite inactive biochemically. With the exception of glucose there is no action on the usual carbohydrates, and even the glucose fermentation is irregular and variable from strain to strain. Coagulated serum is not digested, but gelatin may be liquefied under appropriate circumstances by some strains at least. At temperatures at which gelatin remains solid, growth is sparse and no liquefaction is observed, but if cultures are incubated at 37° C. for 26 to 40 days, the gelatin does not solidify on cooling. Indol is not produced, and nitrates are not reduced to nitrites; small amounts of hydrogen sulfide may be formed. Slight acid production sometimes occurs in milk, with coagulation by the tenth day of incubation and decolorization of the indicator in the lower part of the tube.

The bacillus is but slightly resistant to adverse physical and chemical agents, being readily killed by heat (55° C. for 10 minutes) and bactericidal chemicals. The most effective disinfectants are hypochlorites, iodine, and mercuric chloride. Desiccation experiments have not given uniform results; it is said that cultures dried on threads remain viable for three or four weeks. Pure cultures appear to be more resistant to desiccation than the bacilli in the nasal secretions from diseased animals, for infected discharges are usually sterile within a few days. Cultures die out in four to six weeks but may be maintained by transfer on glycerol agar.

Classification. The relation of the glanders bacillus to other bacteria is by no means clear. In certain respects, such as the occurrence of branching and the favorable effect of glycerol on growth, it resembles the Mycobacteria though it is not acid-fast. In other respects it is related to the Pasteurella species with which it is grouped as Actinobacillus.

Pathogenicity for lower animals. Under natural conditions the horse is chiefly affected, but cases are occasionally observed in the carnivora (cats, dogs, menagerie animals) and in goats and sheep. Swine and pigeons are slightly susceptible. Cattle and house rats are immune. Rabbits and guinea pigs are somewhat susceptible to experimental inoculation, and the hamster and ferret are the most susceptible of the usual experimental animals.⁹²

The route by which the glanders bacillus usually enters the body of the horse has not been clearly determined. The mucous membrane of the nose, especially if slightly abraded, may become the portal of entry, as may the intact conjuctiva, which can be infected by contact with infectious material in two to four hours, sometimes in 30 minutes. Infection by inhalation must be rare, to judge from animal experiments. According to Nocard, infection occurs by way of the alimentary tract in the great majority of cases

Glanders manifests itself in an acute and a chronic form, which run into one another, the latter frequently terminating in an acute attack. The acute form is ushered in usually by a chill and the appearance of a high temperature in advance of any local manifestation. In a few days the mucous membrane of the nose is inflamed and becomes studded with nodules, the lymphatic system becomes largely involved, and edematous swellings appear in various parts of the body. General symptoms become more grave, and death follows in from eight to 30 days. The mule, and especially the ass, suffer commonly from the acute disease. The chronic form is the more usual type in the horse (90 per cent of cases). A great variety of symptoms and lesions have been noted in the latter animal, and the disease pursues most diverse courses in different individuals. The nasal membrane is often affected, and there is a profuse and infectious catarrhal discharge. Cutaneous glanders is known by veterinarians as farcy, the thickenings of the superficial lymphatics being termed "farcy buds" or "farcy pipes." In all forms of glanders there is a tendency to the production of nodules, which soften and pass over into ulcers. The glanders nodule has been considered by some writers to be structurally similar to the nodule formed by the tubercle bacillus (Chap. Thirty), but most observers agree that the former is a degenerative rather than a proliferative formation and that it is radically different from the tubercle.

Experimental inoculation with pure cultures has given positive results not only in the horse, in which the characteristic features of the disease are reproduced, but in guinea pigs, field mice, and other small rodents. The guinea pig is the animal commonly used for diagnostic purposes. Both in the natural and in the experimental infection the bacteria are found chiefly in the nasal secretions and in the contents of the young nodules; in the older ulcers they are relatively few in number. The blood, as a rule, contains glanders bacilli only in acute general infection.

Pathogenicity for man. Veterinarians and others having to do with the care of horses are the most liable to contract glanders. Freshly isolated cultures are highly virulent, and a number of fatal infections have occurred among laboratory workers. The acute form of the disease is the more common in man, most cases terminating fatally within two or three weeks, sometimes within a few days of their inception. As in the horse, the mucous membrane of the nostrils is often, although not invariably, affected. Occasionally the chronic form may

appear and linger for months or even years, with spreading ulceration and other features closely resembling those observed in the horse. Recovery from chronic glanders may take place, or the disease may pass into the acute stage. 55, 84, 99

In man the alimentary tract is certainly not the ordinary channel of entrance; meat from glandered animals has been ingested without resulting infection. Infection probably occurs through abraded skin.

Bacteriological diagnosis. In prebacteriological days chronic glanders in the horse was frequently separated from other diseases only with difficulty and a considerable measure of uncertainty. At present the diagnosis of glanders is greatly facilitated by (1) guinea pig inoculation; (2) the mallein test—(a) subcutaneous, (b) ophthalmic; (3) the agglutination method; (4) the complement-fixation test.

(1) Guinea pig inoculation. A male guinea pig is injected intraperitoneally with fragments of diseased tissue, scrapings from ulcers, or some of the nasal discharge from a suspected animal. A positive reaction is shown by the testicles becoming red and swollen, usually on the second or third day-the Straus reaction. Together with the orchitis (inflammation of the parenchyma of the testicle) there are severe general symptoms which usually culminate in 12 to 15 days. Gravish nodules are often found in the spleen and other internal organs. The test is not absolutely specific, for Kutscher and Nocard have shown that an analogous orchitis may be produced by other organisms besides the glanders bacillus.

(2) The mallein test. Mallein is the concentrated glycerol broth in which the glanders bacillus has grown; it is prepared in the same manner as tuberculin. The mallein reaction consists in a rise of temperature, accompanied by a pronounced local reaction, and in many cases, though not invariably, by more or less profound constitutional disturbances.

(a) Subcutaneous injection of mallein (the size of the dose varying according to the concentration) into a glandered horse is followed by the signs noted above, while in an animal not infected with glanders the temperature is slightly or not at all affected and the general symptoms are absent.

(b) The ophthalmic test consists in the introduction of the mallein, preferably in tablet form, into the conjunctival sac.

The conventional serological reactions. agglutination and complement-fixation.29 tend to give erratic results in that infected animals may show no evidence of antibody formation, and normal serum titers may be relatively high; normal horses, for example, may have agglutinin titers as high as 1:500. Serology is complicated by the fact that the antigenic structure of the bacteria is not established beyond early reports that there is some antigenic heterogeneity.

Chemotherapy. Glanders occurs rarely that little is known concerning its chemotherapy. It has been reported that two chronic cases and six laboratory infections were successfully treated with sulfadiazine.

Immunity and prophylaxis. Permanent immunity to glanders can neither be conferred by an attack of the disease nor produced by artificial means. Nocard fed infectious matter to three horses which had previously recovered from the disease and found that these animals showed no resistance superior to that of a healthy control animal. Similarly, Lobel, Schaar, and Roza⁷⁹ were unable to produce an immunity in guinea pigs or horses by the inoculation of bile-attenuated avirulent bacilli. Chronic glanders may exist for years and is in no wise a warranty against the sudden development of an acute attack.

No very potent or characteristic toxic substance has been obtained from cultures of the glanders bacillus, and attempts at immunization with the products of this organism have been eminently unsuccessful. It is stated by a number of observers that repeated injections of mallein will exercise a curative action upon certain forms of recent infection, but experimentally mallein is without immunizing power. The serums of animals treated with mallein injections and the serums of naturally immune animals such as cattle are, according to most observers, totally devoid of any preventive or curative value. The most that has been accomplished in the way of immunization is a very moderate augmentation of resistance in dogs injected with small nonfatal doses of living cultures.

The experience of Great Britain shows that the disease may be practically eradicated by slaughtering every animal showing clinical signs of glanders, or a positive mallein test, and properly disposing of the carcass.

WHITMORE'S BACILLUS

Melioidosis, a disease of rodents somewhat similar to glanders, is caused by a microorganism variously known as Actinobacillus whitmori, A. pseudomallei, and, more recently, Pseudomonas pseudomallei. The disease occurs in the southwest Pacific area, e.g., in Burma, Thailand, Vietnam, and Indonesia, and in Australia as a disease of sheep, cattle, swine, and horses. It occurs in man in these areas,12,67,111 and has apparently been locally acquired in the

Western Hemisphere.9

Whitmore's bacillus differs from A. mallei in that it is motile, liquefies gelatin, and actively ferments carbohydrates. It grows considerably more rapidly, and its colonies on glycerol agar develop a wrinkled, corrugated surface and are quite different in appearance from those of A. mallei. A second colonial type may be produced, however, which is very similar to the colonies of the glanders bacillus. It is thought by some to be closely related to Ps. aeruginosa in many respects, and strains have been reported which produce pyocyanin. It has been reported that two thermolabile exotoxins are produced, one lethal and necrotizing, and the other lethal only.28,46 A heat-stable endotoxin, closely resembling those of the enteric bacilli and eliciting the Schwartzman reaction, has been described also. 108 An aggressin activity, apparently polypeptide in nature, has been found in sonic lysates of the bacilli; although not toxic itself, when given concurrently with the challenge inoculum it markedly increases mortality.76,80

Melioidosis in man may assume three general forms: an acute septicemic condition with diarrhea, a subacute typhoidal form with pulmonary symptoms and local abscess formation, and a chronic form which may localize in any tissue, including bone, to produce osteomyelitis, as small caseous nodules which may coalesce. It was formerly believed that the case fatality rate in man was very high, 95 per cent or more, but this is presumably applicable only to the untreated, acute form. More recent serological evidence⁹⁶ indicates that the infection may be much more common, and milder, than had been supposed. CONTRACT TO A SCHOOL OF

The tetracyclines appear to be the most active of the chemotherapeutic agents when tested in vitro, 95 but the experimental disease in mice is most effectively treated with chloramphenical or sulfonamides, the tetracyclines requiring extended periods of treatment to give 70 per cent or better cures. 49

Transmission of the infection among guinea pigs by biting insects, mosquitoes, and fleas, has been reported. Rodents usually die within a short time from septicemia, and the lesions appear as small nodules superficially resembling tubercles. Guinea pigs and rabbits are highly susceptible to inoculation, and the Straus reaction is produced.

ACTINOBACILLOSIS

Actinobacillus lignieresi. In 1902 Ligniers and Spitz isolated a nonmotile, nonbranching, nonacid-fast, gram-negative bacillus from the lesions of a disease of cattle which closely resembles actinomycosis and with which it is frequently confused. The microorganism is known as A. lignieresi and the disease as actinobacillosis. Originally found in Argentina, it appears to be relatively common and has been recognized in Europe and in this country.

Granules, very similar to but smaller and more numerous than the "sulfur granules" of actinomycosis, are found in the thick pus from the lesions. These granules or colonies contain clubs radially arranged about a center which is composed of detritus and gram-negative bacilli. The microorganism is pleomorphic in cultures; diplococci and slender rods are found in smears from cultures in liquid mediums, while long curved forms are present in deep colonies in agar mediums. The bacilli are $0.4~\mu$ in diameter and 1 to 15 μ in length.

Surface colonies on laboratory mediums are small, 0.5 to 1 mm. in diameter, smooth, glistening, convex, bluish white, and delicate in appearance. In liquid mediums, such as serum-glucose-broth, the growth consists of small grayish granules adhering to the sides of the tube and readily broken loose by shaking; the broth does not become turbid. The microorganism appears to be a strict parasite and grows very poorly or not at all except in mediums containing serum or whole blood. It is an obligate aerobe though primary cultures are often more successful in fluid mediums or serum-glu-

cose-agar stab cultures, especially when incubated in an atmosphere containing 10 per cent carbon dioxide. Cultures grow up in 24 hours at 37° C.; growth is very slight at 20° C. Dextrose, lactose, sucrose, maltose, raffinose, and mannitol are fermented; the fermentation of xylose is irregular. Arabinose, dulcitol, salicin, and inulin are not fermented. Very small amounts of indol are produced, coagulated serum is not liquefied, and litmus milk is usually unchanged but is sometimes slightly acid.*

As indicated above, the disease in cattle closely resembles actinomycosis, differing in that the bones are seldom affected and the lesions are found in the soft tissues, the regional lymphatics being commonly involved; the subcutaneous tumors break down in time to form abscesses. The subcutaneous lesions respond to surgery, but infection of the tongue, "wooden tongue," is often fatal. The disease has been observed to occur in both epizootic and sporadic form. It is readily reproduced by inoculation of cattle, but the microorganism is only feebly pathogenic for the usual experimental animals; massive intraperitoneal inoculation of the guinea pig produces a scrotal reaction similar to the Straus reaction. A very few probable cases of human infection have been reported.40

Actinobacillus actinoides. 68 This microorganism was originally isolated from the lungs of calves suffering from chronic pneumonia and later from a similar affection in white rats. It is not pathogenic for other experimental animals, and, in fact, its etiological relationship to the observed disease is questionable.

Actinobacillus actinomycetem - comitans. This bacillus has been observed and isolated from human cases of infection with Actinomyces bovis, first by Kligler in Germany in 1912, and by Comstock in England in 1920, and Bayne-Jones in 1925 in this country. It occurs as densely packed, gram-negative coccobacilli in contrast to the gram-positive actinomycete mycelium in the interior of the sulfur granules. It has never been reported in bovine actinomycosis, and the significance of its presence is open to question.

Challenger in

^{*}There is some discrepancy in the literature on these points.

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Chapter Twenty-six

THE HEMOPHILIC AND RELATED BACTERIA

The hemophilic bacteria are characterized by a nutritional requirement for constituents of fresh blood, especially hemoglobin and certain related compounds, or the X factor, and/or a requirement for the heat-labile V factor which may be replaced by NAD or NADP. Not all bacteria grouped under the genus Hemophilus have these nutritive requirements, notably the pertussis bacillus, the Morax-Axenfeld bacillus and Ducrey's bacillus, but they are otherwise similar. They are related antigenically to certain of the Brucella species, such as Br. bronchiseptica as noted elsewhere and lines of distinction may be difficult to draw. The pertussis bacillus has, in fact, been placed in a separate genus, Bordetella, by some.

The Hemophilic Group

SPECIES	GRO' REQUIR	HEMOL-	
	X FACTOR	V FACTOR	1313
H. influenzae	+	+	
H. hemolyticus	+	+	+
H. parainfluenzae		+	±
H. suis (influenzae			
suis)	+	+	
H. canis (hemoglo- binophilus)	4		
H. pertussis			+
H. duplex (Morax-Axenfeld)	÷.		.
H. ducreyi			• • •

HEMOPHILUS INFLUENZAE (PFEIFFER'S BACILLUS)

Hemophilus influenzae was isolated by Pfeiffer in 1892, and until the early 1930's was regarded by many as the etiological agent of epidemic influenza. Influenza has, however, been shown to be caused by a virus, and the name influenzae has no etiological significance.

Morphology and staining. Pfeiffer's bacillus is one of the smallest pathogenic bacteria, rarely exceeding 1.5 μ in length and 0.3 μ in thickness. The ends of the cell are rounded, capsules are not generally observed but are present in smooth cultures, spores are not formed, and the bacillus is nonmotile. There is a marked tendency to produce threads and other anomalous forms in culture which is, to some degree, a characteristic of strains. Some workers have attempted to differentiate varieties on the basis of morphology, but there is a continuous series of types, ranging from precoccobacillary dominantly forms predominantly longer bacilli and threads, and no sharp distinction can be made. There is a tendency, however, to regard the coccobacillary forms as "typical" and the longer forms as "atypical"; the typical form appears to predominate in strains isolated from pathological processes.

On blood agar the colonies of Pfeiffer's bacillus are very small, rounded, discrete, and transparent and may reach the size of a small pinhead. If the culture is contam-

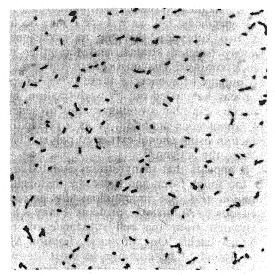


Figure 139. Hemophilus influenzae, pure culture. Note the variability from coccoid to bacillary form and the presence of longer filaments. Fuchsin; × 1050.

inated with other microorganisms, especially Staphylococcus aureus, the colonies are considerably larger, more opaque, and of a grayish white color, and develop most luxuriantly in the neighborhood of the foreign colony, a phenomenon termed the satellite phenomenon.

These bacilli are somewhat more difficult to stain than most bacteria: Löffler's methylene blue for five minutes or dilute (1:10) carbol-fuchsin for 10 minutes is satisfactory. They are gram-negative.

Physiology. One of the more fastidious bacteria, Pfeiffer's bacillus requires, as noted above, the presence of blood in the culture medium. It has been found that two substances present in such blood mediums are necessary to the growth of this bacterium; one, designated the "X factor," is heat-stable and associated with hemoglobin, and the other, the "V factor," is heat-labile and is found in yeast and various vegetable extracts as well as in whole blood. The satellite phenomenon noted above is due to the formation of the V factor by other bacteria and its diffusion into the medium from the colony.

The X factor is replaceable by hemoglobin or hematin. The iron protoporphyrin can be replaced with certain other iron porphyrins, but not by porphyrins such as meso-, hemato-, deutero-, and coproporphyrins. These last suppress growth by a competitive inhibition,28 and such activity is eliminated by methylation of the propionic acid side chains.24 It seems that these or related compounds are required for respiration-some strains do not require it for anaerobic growth. It has been suggested that hematin is required for the synthesis of catalase; hematin can be replaced by cysteine which would reduce peroxide and make catalase unnecessary.20

The V factor may be replaced by coenzyme I or coenzyme II but not by nicotinic acid or its amide. Apparently the whole coenzyme molecule must be supplied, and it is assumed that the thermolabile substance is of this nature. Some strains require 1.4diaminobutane, the active residue available in putrescine, spermine, and spermidine. Other growth requirements include pantothenic acid, thiamine, and uracil, and some strains require purine also.26 Some strains of H. influenzae, and H. parainfluenzae, have been grown in defined mediums.9, 58

A number of mediums have been devised for the cultivation of the influenza bacillus. Avery's oleate hemoglobin agar, prepared by the addition of sodium oleate and a suspension of erythrocytes to an infusion agar base, is one of the best of these, for not only is the growth of H. influenzae enhanced, but the growth of streptococci and pneumococci and some other bacteria present in the sputum and nasal mucus is inhibited. The influenza bacillus grows luxuriantly chocolate agar, prepared by the addition of fresh blood to hot (90° C.) infusion agar, and heavy growths for agglutination and other purposes may be obtained, but this medium does not differentiate and hence is not especially suitable for primary isolation. Fildes' agar, an infusion agar base to which has been added a peptic digest of blood, supports good growth of the influenza bacillus and is used especially by British workers.

No growth occurs on gelatin. Milk, containing blood, is rendered slightly alkaline by some strains. Nitrate is reduced to nitrite. Some strains-about 50 per centform indol. Fermentation reactions are variable, some strains being inactive while others ferment dextrose and other carbohydrates. Some strains are hemolytic while others are not. The hemolytic strains of H. influenzae do not appear to be clearly marked from the nonhemolytic strains by any other differential characters, since indol production and carbohydrate fermentation occur in both groups.

The influenza bacillus shows little resistance toward external conditions. It is readily killed by drying, and pure culture suspended in water and then dried on silk threads dies within 24 hours but in dried sputum viability persists somewhat longer, but not, as a rule, beyond 48 hours. The bacilli are readily killed by disinfectants. Even under favorable conditions artificial cultures soon die out, and, in order to preserve vitality, subcultures must be made every four or five days; chocolate agar is suitable for maintaining stock cultures.

Varieties. Subdivisions of *H. influenzae* have been made by a number of workers, but whether such varieties deserve the dignity of species standing is open to question. The morphologically "typical" and "atypical" forms have been noted above, but these are not regarded as separate species. Other distinctions which have been made on the basis of hemolysis and nutritive requirements may be summarized briefly:

(1) The hemolytic and nonhemolytic varieties. The nonhemolytic form is designated *H. influenzae* by Bergey and the hemolytic form *H. hemolyticus*. Both require the X and V nutritive factors. These are generally regarded as a single species, *H. influenzae*.

(2) The swine influenza bacillus, *H. influenzae suis* or *H. suis*, which closely resembles Pfeiffer's bacillus except that it is relatively inert biochemically and differs immunologically. This bacterium, in association with a virus, is causally associated with swine influenza. Both X and V factors are required for growth.

(3) The para-influenza bacilli, *H. parainfluenzae*, which closely resemble *H. influenzae* except that only the V factor is required for growth and these bacteria may be cultivated on agar containing serum or ascitic fluid. Although these bacilli are defined as nonhemolytic, hemolytic strains showing the same nutritive requirements are found.

(4) H. canis (H. hemoglobinophilus, H. hemoglobinophilus canis), found in the preputial secretions of dogs. It closely resembles H. influenzae except that it requires only the X factor for growth.

Variation and antigenic structure. As tested by direct agglutination, *H. influenzae* is antigenically heterogeneous, and rough and smooth forms occur in which the smooth form is encapsulated. By means of the agglutination test the encapsulated bacilli are found to fall into six immunological types, designated a, b, c, d, e, and f, and the antigens responsible are specific polysaccharides.³⁵ One of these, the type-specific substance of type b, is a polymer of ribose phosphate with a structure analogous to that of pentose nucleic acids in which the purine and pyrimidine portions are replaced

by a second polyribose phosphate chain in 1:1' glycosidic linkage.⁵⁹ Type e may occur as two subtypes, the one as occasional freshly isolated strains, and the other in stock strains.⁵⁷ Diagnostic antiserums may be prepared for each of these types.¹ Typing may be carried out by agglutination, by specific capsular swelling in the presence of homologous antibody, or as a precipitin reaction using phenol-extracted polysaccharide as the antigen.³⁴

It appears that many strains isolated are in the rough form, and these, by the agglutination test, are immunologically heterogeneous. Antigenic proteins may be extracted from the cell substance of influenza bacilli. One of these, fraction M, was found to be common to most strains of H. influenzae, indicating an immunological homogeneity demonstrable by precipitin tests with appropriately prepared antigens. It will be clear that the antigenic structure of H. influenzae is by no means fully understood as yet.

The influenza bacilli appear to be immunologically related to certain of the pneumococcus types; type a shows cross-reactions with pneumococcus type 6b and type b cross-reacts with pneumococcus type 6 and type 29.

The S-R dissociation is to some degree reversible by growth in the presence of anti-R immune serum. The relation of virulence to this dissociative change is not known.

Toxins. As in the case of many other bacteria, the cell substance of the influenza bacillus is toxic to experimental animals, mice in particular, upon parenteral inoculation. Toxic substances are produced in fluid cultures, are filterable, and may appear in appreciable quantities after six to eight hours' incubation. Relatively large quantities of the filtrate (2 to 4 ml.) are necessary to produce death in rabbits, and it is probable that a true exotoxin is not formed.

Pathogenicity for man. The pathogenicity of Pfeiffer's bacillus for man is shown by the occurrence of cases of meningitis, mostly in infants and of a high fatality, in which H. influenzae is found in pure culture in the cerebrospinal fluid. The organisms persist in nasopharyngeal carriers, with a frequency of perhaps 2 to 3 per cent. As in meningococcal infections, the carrier rate is higher when disease occurs, as in an infected household. H. influenzae meningitis is the fourth

commonest form of purulent meningitis; it occurs most often the second six months of life, and the case fatality rate in untreated cases is between 90 and 100 per cent. In one series of 758 cases of meningitis occurring between 1930 and 1953, *H. influenzae* type b was the causal agent in 128.³¹ Occasional cases of otitis media, appendicitis, sinusitis, and other localized infections may be caused by this bacterium.

The influenza bacilli are frequently present in infections of the respiratory tract and are found on autopsy in pneumonic lesions under conditions where its pathogenicity is clearly indicated. They have also been associated with respiratory disease in premature infants,14 and serological studies suggest that they may be a factor in chronic bronchitis. 21 Whether they are present as a primary or secondary invader in these cases is more uncertain. Their common occurrence in diseases such as measles, whooping cough, and tuberculosis has been taken to suggest that their growth on human tissue is favored by the presence of other infecting agents. It seems probable that in respiratory infections H. influenzae is not the primary agent.

The relation of this bacillus to influenza is very likely that of a secondary invader to the initial virus infection. It has been found that in double infections of the chick embryo with influenza virus, the pathogenicity of *H. influenzae* is enhanced though that of the virus is not affected.⁸ It is frequently but not invariably present in cases of influenza and, of course, occurs in the absence of this disease. In the case of swine influenza, the influenza bacillus is, in association with a filterable virus, causally related to the disease.

Chemotherapy. Chemotherapy has been of greatest interest in connection with H. influenzae meningitis which, in untreated cases, is almost always fatal. Penicillin is not effective, but sulfonamides, streptomycin, and chlortetracycline, sometimes in combination with antiserum, may be highly effective.7, 11, 36 In one series of cases studied,31 for example, treatment with sulfonamide alone gave 15 per cent survival and in combination with antiserum 83 per cent survival, but complications including mental retardation, blindness, hydrocephalus, and convulsions occurred in 20 per cent of survivors. The rise in recovery rate, due to more effective therapy, has resulted in an increase in such complications, i.e., in patients who would otherwise have died. A combination of sulfonamide, streptomycin, and antiserum gave a case fatality rate of 7.4 per cent, and of chlortetracycline and sulfonamide 4.3 per cent, but with the same incidence of complications. Treatment with sulfonamide, streptomycin, and chlortetracycline, occasionally reinforced with chloramphenicol, reduced the case fatality rate to 3.2 per cent and the incidence of complications in survivors to 5 per cent. H. influenzae is not sensitive to penicillin, and in fact the first use to which this antibiotic was put was that of a selective agent incorporated in isolation mediums.

Pathogenicity for lower animals. Except in swine influenza, the influenza bacillus is probably not a natural pathogen of lower animals. Upon intraperitoneal inoculation into laboratory animals-mice, guinea pigs, and rabbits-large doses of these bacteria produce death within one or two days. Whether this is an actual invasion rather than a toxemia is uncertain: the bacilli may be found in the peritoneal exudate but usually not in the heart's blood, and petechial hemorrhages may be observed scattered over the peritoneum and, sometimes, the pleura. Certain strains produce a fatal infection in mice on intracerebral inoculation. As tested by intraperitoneal inoculation, the virulence of H. influenzae varies greatly from strain to strain; the virulent strains are in a minority, and strains from influenzal meningitis are generally among the more virulent.

Other hemophilic bacteria have been described in connection with diseases of lower animals. H. canis, for example, has been found in association with inflammation of the prepuce in dogs but is apparently harmless. Other hemophilic bacilli such as H. bovis, H. gallinarium, H. muris, and H. ovis have been isolated from lower animals. Their relationship to H. influenzae and other better-known species is not clear.

THE KOCH-WEEKS BACILLUS

A small bacillus, first observed by Koch in 1883 in a series of eye inflammations in Egypt, was successfully cultivated by Weeks in New York in 1887, and is now recognized as the cause of a world-wide and highly

contagious form of conjunctivitis sometimes known as pink-eye. The disease is especially prone to occur in tropical and subtropical climates, and may assume epidemic form.⁵¹

This microorganism is a gram-negative, nonmotile, nonencapsulated facultative aerobe, and sometimes shows bipolar staining. It requires the X and V factors for growth. There is no hemolysis on blood agar, and the colonies are small and translucent with a bluish tinge in transmitted light.

This bacillus is closely related to *H. influenzae* and has been regarded by many as identical with it. Pittman and Davis⁴² have found, however, that strains of this organism make up a closely related but not homogeneous serological group, distinct from the heterogeneous nontype-specific *H. influenzae*, and differentiate it as *H. aegyptius*. Antigenic components are, however, shared with *H. influenzae*, and are demonstrable by the gel-diffusion method,⁴⁰ and a high degree of genetic homology between the two is indicated by the DNA-mediated transfer of antibiotic resistance.³³

It produces conjunctivitis in man but not in laboratory animals. It has low virulence for mice when inoculated in mucin suspension, a high virulence for the eight day chick embryo, but not for the 12-day embryo.

HEMOPHILUS PERTUSSIS (BORDETELLA PERTUSSIS)

Bacilli resembling H. influenzae were reported by early observers as occurring in a large proportion of cases of whooping cough. Although there are minor differences in the descriptions of these organisms as given by different observers, the cultural and morphological characters are essentially similar, and there seems little doubt that Spengler, Jochmann and Kraus, Wollstein. and Davis discovered the same bacillus. More definite results were obtained by Bordet and Gengou in 1908, who found in the bronchial exudate from patients with whooping cough a characteristic short oval bacillus which grew feebly on a special medium they devised. Earlier named Bacillus pertussis, this microorganism is now known either by that name or as H. pertussis or, more casually, as the Bordet-Gengou bacillus.

Morphology and staining. The Bordet-Gengou bacillus is a small ovoid rod from 1.0 to 1.5 μ in length and 0.3 to 0.5 μ in breadth. The majority of the bacteria occur singly, although they may occasionally be seen in pairs end to end; chains do not occur in smears of bronchial exudate, but short chains may be seen in cultures in liquid mediums. This morphology is relatively constant, and there is not the tendency to the formation of thread-like and other aberrant forms which is exhibited by the influenza bacillus. H. pertussis is nonmotile and nonspore-forming, and the smooth form is encapsulated.

On the Bordet-Gengou medium the colonies are smooth, raised, and glistening, with a metallic or pearl-like luster, and are larger and more opaque than those of the influenza bacillus; 48 to 72 hours' incubation is required for their appearance and development. Upon further incubation they acquire a slight brownish color. A mucoid substance is abundantly produced by the culture, and the growth is sticky and tenacious. On blood agar the colonies are surrounded by a narrow zone of hazy hemolysis.

The bacilli stain with a little difficulty and, as in the case of the influenza bacillus, methylene blue or dilute carbol fuchsin must be applied for five to 10 minutes; carbol toluidine blue is recommended by some workers and stains the bacilli a lilac color. A tendency to bipolar staining may be observed. They are gram-negative.

Physiology. H. pertussis is difficult to cultivate upon primary isolation, and the Bordet-Gengou medium upon which it grows readily consists of 1 per cent glycerol agar on glycerol broth made with macerated potato and added to an equal volume of human or rabbit blood. By repeated passage on mediums containing less and less blood, however, it may be acclimatized and will, in time, grow, although sparsely, upon ordinary nutrient agar. It does not, therefore, require the V and X factors that are essential to the development of the hemophilic bacteria. The optimum temperature is 37° C., and the bacillus is aerobic and facultatively anaerobic.

Growth with the maintenance of immunogenic potency of phase I (see below) for vaccine production is a matter of practical importance. A number of casein hydrolysate mediums have been devised^{53, 55} for this purpose, and the inclusion of charcoal in beef heart agar tends to preserve immu-

PERTUSSIS 583

nogenic potency.³⁸ Starch is required in such mediums.

H. pertussis is biochemically inactive. It does not form indol, does not reduce nitrates, and does not ferment any sugars. Its resistance is slight and of the same order as that of the influenza bacillus. It is killed by exposure to 55° C. for 30 minutes.

Toxins. As in the case of Pfeiffer's bacillus, the cell substance of H. pertussis is toxic upon parenteral injection into experimental animals and to about the same degree. Endotoxin may be separated as a watery extract of disintegrated cells. There appear to be two fractions, one heat-stable and the other heat-labile and inactivated in 30 minutes at 56° C. On intravenous inoculation in rabbits the heat-stable fraction produces hyperglycemia and the heat-labile fraction hypoglycemia. Intratracheal inoculation in rabbits produces an edematous reaction followed by a lymphocytic infiltration about the blood vessels and bronchi which is reported to be similar to the changes produced in the lung in whooping cough. The endotoxin of a variant strain has been prepared by fractionation of sonic lysates⁵⁶ by extraction of lyophilized bacilli with calcium chloride, followed by methanol precipitation in the cold,47 and by ion exchange chromatography.5

Variation and antigenic structure. Unlike the influenza bacillus, H. pertussis is generally in the smooth state when isolated from the body on an optimal medium and is immunologically homogeneous; i.e., the virulent form occurs only as a single antigenic type,⁵⁴ phase I. Culture strains fall into four immunological groups which are designated phases I, II, III, and IV. Although not obviously rough, phases III and IV are somewhat rougher in appearance and less stable in saline suspensions than the bacilli of phase I. The S-R dissociation of H. pertussis occurs readily on culture on artificial mediums, even on blood agar, and it seems probable that these phases do not represent distinct immunological types but rather successive stages in the S-R transformation. As indicated above, virulence and immunogenicity may be maintained by cultivation on charcoal agar.43

The S-R change, of which the above phases represent the early stages, proceeds to the obviously rough state with consequent alterations in colonial morphology and loss of virulence.

As indicated elsewhere, H. pertussis is

immunologically related to Brucella bronchiseptica. Atypical bacilli isolated from a small proportion of cases of whooping cough have been designated H. parapertussis. These bacilli differ from H. pertussis in that they grow readily on ordinary nutrient agar upon isolation and produce an alkalinity in litmus milk in two to four days. Immunologically, they are related to both H. pertussis and Br. bronchiseptica through common O antigens, but are differentiated by capsular, or K, antigens.^{2, 28}

Pathogenicity for man.27,50 Whooping cough is widespread throughout the entire world, and it is estimated that 95 per cent of the population suffer from it, in typical or atypical form, at one time or another. The typical disease is commonest in the lower age groups and is much more serious in children than in adults. While the mortality has been steadily reduced, its relative importance as a cause of death under five years has increased progressively, and now it is exceeded in importance in this age group only by gastroenteritis, pneumonia and bronchitis, and tuberculosis. It occurs in three stages, each lasting about two weeks. The catarrhal stage begins with a mild cough that progresses in severity to a paroxysmal stage characterized by rapid consecutive coughs and the deep inspiratory whoop. In the convalescent stage the number and frequency of paroxysms gradually decrease, and recovery is uneventful except that bronchial and lobar pneumonia and otitis media occur with some frequency as complications, especially in children. The bacilli are present in large numbers in the respiratory tract in the catarrhal, and most contagious, stage, and gradually disappear until they are rarely found after the fourth week.

Although certain features of whooping cough may be approximately reproduced in experimental animals, the mechanism by which the microorganisms produce the disease in man is not altogether clear. The characteristic lymphocytosis and the leucocytic exudate into the lumen of the trachea and bronchi have been regarded as evidence of the activity of the endotoxins. The bacilli are also present in large numbers, especially in the catarrhal stage, between the cilia and possibly interfere mechanically with ciliary action. The localization of the bacteria and especially the persistence of the paroxysmal cough after the bacteria are no longer detectable, however, tend to set whooping cough somewhat apart from the other bac-

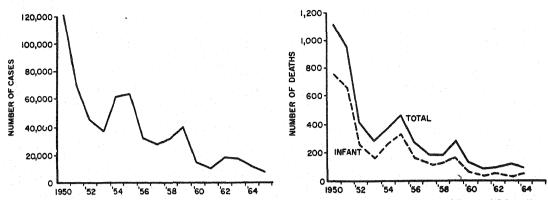


Figure 140. Pertussis cases and deaths reported in the United States, 1950-1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

terial respiratory infections. The observation that in the mouse either parenteral inoculation with vaccine or intranasal infection results in a remarkable increase in sensitivity to histamine (see below) suggests that some form of tissue sensitivity, of which increased histamine sensitivity may be a manifestation, plays a part in the pathogenesis of the disease.

Pathogenicity for animals. Naturally occurring infections of lower animals with H. pertussis do not occur and, in general, it is of variable and usually low virulence for experimental animals. The developing chick embryo may be infected, with reproduction of the cellular infiltration and necrosis of the bronchi seen at autopsy of fatal cases of the human disease,48 and the bacilli may be grown in tissue culture. 10 Both the rat and the mouse may be infected by intranasal inoculation to produce interstitial pneumonia with leucocytic infiltration around the vessels and bronchioles and mucous secretion on the bronchial epithelium.49 The mouse may be infected by intracerebral inoculation; smooth strains are the more virulent and the most virulent strains have an LD50 of 100 to 1000 bacilli by this route. Intracerebral infection of the mouse is used routinely in the standardization of the immunogenic potency of vaccines in this country; while it would appear to be quite unrelated to the human disease, Pittman⁴¹ has found that there is a reasonable correlation between virulence as tested by this method and by intranasal inoculation of the mouse.

Bacteriological diagnosis.³² The bacillus may be isolated by the cough plate method in which the open plate is held 4 to 5 inches

from the mouth and exposed to one or more explosive coughs. Nasopharyngeal swab cultures give a higher proportion of positive cultures than the cough-plate method, and the pernasal swab a higher proportion of positive cultures than the postnasal swab.³⁷ The swab, on thin, flexible, copper wire, is passed through a nostril into the nasopharvnx and left there for two or three coughs before withdrawal, and cultured on Bordet-Gengou medium. Sputum culture is not particularly satisfactory. Colonies of H. pertussis are larger and more opaque than those of the influenza bacillus; it may be differentiated further by hemolysis on blood agar and growth in the absence of the X and V factors.

Chemotherapy.^{25, 44} The chemotherapy of whooping cough is not as successful as that of many of the other infectious diseases, but the broad-spectrum antibiotics, chloramphenicol and the tetracyclines, have been found to reduce the duration and severity of the disease significantly in many instances, and occasional dramatic results have been reported. The sulfonamides and penicillin are ineffective.

Epidemiology.²² An upper respiratory infection, whooping cough is transmitted by means of the secretions of the mouth and nose, to some extent through towels, hand-kerchiefs, hand-to-hand contact, and the like, and undoubtedly to a great extent by droplet infection. Undiagnosed and atypical cases may play an important part in the dissemination of the disease. The age incidence is marked, with 96 per cent of the deaths in children under five years of age, and there is no doubt that whooping cough is one of

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585

the most important killing diseases of childhood. There appears to be some racial difference in susceptibility, since the Negro death rate is considerably higher than that of the whites. In contrast to most other diseases the incidence in females is somewhat higher than that in males and in children in higher economic groups. The seasonal incidence shows a drop during late summer and early fall, with a prolonged peak in the later winter and spring. The disease tends to recur in periodic epidemic waves, presumably a consequence of the accumulation of a new crop of susceptibles.

Immunity. Recovery from whooping cough is accompanied by the development of immunity. Second attacks occur only infrequently in children and then are very mild; in older people second attacks are more severe. Complement-fixing antibodies are formed but do not appear until the third or fourth week of the disease and, consequently, are of limited diagnostic value.

Under experimental conditions are three kinds of response to the bacilli. There is a very rapid development, usually within a few hours, of a refractory state in which the animals are highly, and specifically, resistant to challenge inoculation, which persists for a week or more;^{17, 18} it is considered to be in the nature of an interference phenomenon. Second, as a consequence of inoculation with vaccine, or infection, mice become extremely sensitive to the action of histamine.29 Such sensitized animals show an enhanced susceptibility to anaphylaxis, a local cutaneous reaction as well as systemic anaphylaxis.30 Finally, the usual antibody response, with the presence of protective, complement-fixing, etc., antibody demonstrable in the serum occurs also. The protective antigen is separable from the toxin; it has been prepared in soluble form by partial deoxycholate lysis of the bacteria⁶ and purified from lysate by starch block electrophoresis.39 In the latter preparation the protective antigen and the histamine-sensitizing substance were not separable, although separation of the two has also been reported.13

Prophylactic inoculation. The early attempts to use vaccines of *H. pertussis* were not uniformly successful. More encouraging results were obtained by Madsen and other Danish bacteriologists in the Faroe Islands. In the United States Sauer and his colleagues have prepared vaccines

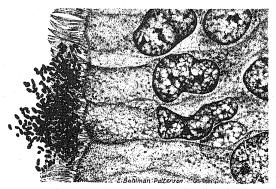


Figure 141. Whooping cough. Minute bacilli present in masses between cilia of two cells lining the trachea. About \times 1500.

of freshly isolated, virulent strains which appear to be effective in reducing the incidence and severity of the disease. It is of primary importance that the vaccine consist of the smooth bacilli of phase I, and earlier reports of the inefficacy of active immunization are probably attributable in part, at least, to the use of other than the smooth form of the microorganism. Immunogenic potency of vaccines requires constant assay and control: in the United States a standard test, using intracerebral challenge in the mouse, is used, and similar control procedures are used by others.3 Alum-precipitated vaccine seems to be effective also, but it is not known whether it is superior to plain vaccine.

Pertussis vaccine may be successfully combined with diphtheria toxoid and tetanus toxoid to allow immunization against the three diseases simultaneously; available evidence indicates that the immune response is equal to that obtained with separate antigens. In quadruple vaccines, containing inactivated poliomyelitis antigen also, the immunogenic potency of the pertussis component deteriorates, presumably as a consequence of the presence of a protease derived from the monkey kidney tissue used in the production of the poliovirus component. It is generally agreed that the disease is appreciably milder in immunized children.

With the demonstration of the endotoxins of *H. pertussis*, considerable interest has attached to the possible role of anti-endotoxin in immunity to the disease. The toxicity appears to be neutralized with appropriately prepared antiserum, and studies with experimental animals suggest that

antitoxic antibody may be significant in the immunity.

THE MORAX-AXENFELD DIPLOBACILLUS (HEMOPHILUS DUPLEX)

A small bacillus, described independently by Morax and by Axenfeld in 1896 and 1897, is responsible for infections of the conjunctiva and cornea in man and is known as the Morax-Axenfeld bacillus or *B. lacunatus* (from the lacunae of liquefaction produced by growth on coagulated serum). It has been grouped with the hemophilic bac-

teria as H. duplex.

The short rods, 1 μ by 2 to 3 μ , frequently appear end to end in pairs or short chains. They are nonmotile, nonspore-forming and gram-negative. They do not grow on the ordinary nutrient mediums, potato, milk, or gelatin but require the presence of serum, ascitic fluid, or blood in the culture medium. They ferment few if any carbohydrates and do not form indol. Coagulated serum is liquefied. They die out within a day or two at room temperature but may survive for weeks in culture in the incubator. It has been reported that hemolytic and nonhemolytic types occur which may be differentiated immunologically.

So far as is known this microorganism is pathogenic only for the human eye. The inoculation of experimental animals is without effect, but the instillation of the bacilli onto the conjunctival sac of man results in the development of blepharo-conjunctivitis, either chronic or acute, and severe inflammation of the cornea may be produced. Treatment with 0.25 per cent zinc sulfate solution is specific and produces a rapid cure, while silver salts are without effect.

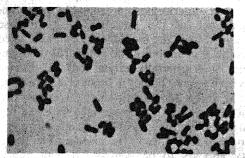


Figure 142. Hemophilus duplex (Morax-Axenfeld bacillus); pure culture. Gram stain; × 2400.

Chlortetracycline is reported to be highly effective when used as a 1 per cent ointment, and streptomycin is effective also. The disease is widely distributed and has been reported in Europe, Africa, and North America.

DUCREY'S BACILLUS (HEMOPHILUS DUCREYI)

Soft chancre or chancroid is a venereal disease transmitted by direct contact. The lesions, which are on the genitals or adjacent areas, are irregular ulcers which differ from the hard or Hunterian chancre—the primary lesion of syphilis—in that they are not indurated. Unlike syphilis, the infection remains localized, spreading no further than the neighboring lymphatics, which may become swollen to form secondary buboes in the groin.⁴

The bacillus was found by Ducrey in 1890 in the purulent discharge from the lesion, and by the inoculation of the skin of the forearm he was able to transmit the disease through 15 generations. The microorganism was obtained in pure culture by Besancon, Griffon, and le Sourd in the same

vear.

Ducrey's bacillus is a short rod 1 to 1.5 μ in length and 0.6 μ in breadth. In smears it is generally found to be ovoid, and there is a tendency to occur in end-to-end pairs or in short chains; in broth culture longer chains may be observed. It is nonspore-forming and nonmotile. The bacillus not infrequently stains irregularly and bipolar staining may be observed. It is stained by the usual aniline dyes and is gram-negative.

The bacillus will not grow on the ordinary laboratory mediums and requires the addition of serum or, preferably, blood.⁴⁵ The X and V factors alone are not sufficient, and additional factors present in either serum or erythrocytes are required. The small, grayish, glistening colonies appear in blood agar in 24 hours and, after two to three days' incubation, show a narrow zone of hemolysis. The bacilli may be cultivated from the chancroid by inoculating tubes of fresh (not more than three to five days old) rabbit blood. Smears made at the end of 24 to 48 hours' incubation will show the characteristic tangled chains of gram-negative bacilli. These bacteria cannot be identified

in smears from the chancroid because of a tendency to aberrant morphology. The bacilli may be isolated and cultivated on the chorioallantois of the developing chick embryo and appear to have little pathogenicity for the embryo. Rabbits and monkeys may be infected by intradermal inoculation of pure cultures, but subcutaneous, intraperitoneal, and intravenous inoculation is without effect. Infected animals develop a hypersensitivity but no immunity to reinfection. ¹².

There is little or no immunity. The chancroid is frequently multiple and is auto-inoculable. A hypersensitivity develops, which is demonstrable as a reaction to the intradermal inoculation of killed bacilli and which persists for many years. The disease may be successfully treated with the common drugs with the exception of penicillin; chlortetracycline is regarded by many as the drug of choice, and streptomycin has given excellent results.

Hemophilus vaginalis. Another hemophilic microorganism has been described as occurring in association with, and possibly in etiological relation to, human vaginitis.¹⁹ Like the other members of the group, H. vaginalis is a small, nonencapsulated, nonmotile gram-negative bacillus. It is somewhat more exacting in its nutritive requirements than the other Hemophilus species, is relatively inactive biochemically, and the delicate growth is facilitated by incubation in increased carbon dioxide tension.16, 46 Penicillin may be incorporated into the medium $(10\mu/\text{ml.})$ for primary isolation from contaminated specimens. It is reported to be clearly distinguishable serologically, by growth requirements, and by cultural characteristics.15

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THE SERVICE SERVICE SERVICES

Chapter Twenty-seven

PSEUDOMONAS; LACTOBACILLUS; LISTERIA; ERYSIPELOTHRIX; BACTEROIDES; STREPTOBACILLUS; BARTONELLA; MYCOPLASMA; DONOVANIA

Pseudomonas aeruginosa (pyocyanea)

The genus Pseudomonas³⁹ includes some 30 species which are found, for the most part, in water, soil, and wherever organic matter is decomposing. The fluorescent bacteria are members of this genus, *Pseudomonas fluorescens* being the gelatin-liquefying form and *Ps. nonliquefaciens* the nonliquefying form. The elaboration of blue pigment by *Ps. syncyanea* results in "blue milk." One species, *Ps. septica*, is the cause of a disease of caterpillars, another, *Ps. reptilovorus*, produces disease in certain reptiles.

The best known species of Pseudomonas, however, and the only one that is pathogenic for man, is *Ps. aeruginosa*, which is also commonly known as *Ps. pyocyanea*. The blue or blue-green stains that sometimes appear upon surgical dressings long ago attracted attention, and even before the cause of the phenomenon had been discovered Fordos studied the pigment in 1860. Gessard found, in 1882, that the pigment was the product of a specific microorganism, *Ps. aeruginosa*, which he isolated in pure culture.

Morphology and staining. The cells of Ps. aeruginosa vary considerably in size and proportion but appear usually as small, slender rods, 1.5 to 3 μ long and 0.5 μ broad, frequently united in pairs and short chains. There are one to three polar flagella, and the

bacterium is actively motile. Neither capsules nor spores are formed. The colonies are large and spreading, the edges are irregular, and the consistency butyrous. The bacilli stain readily with the usual aniline dyes and are gram-negative.

Physiology. Ps. aeruginosa grows readily on all the ordinary culture mediums and most rapidly at a temperature of 30° to

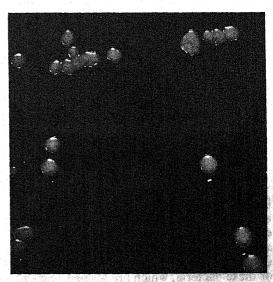


Figure 143. Colonies of *Pseudomonas fluorescens* on nutrient agar, 24-hour culture. × 3.

37° C. Aerobic conditions are required, although there is some growth under anaerobic conditions. Gelatin is rapidly liquefied, hydrogen sulfide is produced, and indol is not formed (false positives are sometimes given with Ehrlich's reagent, but not with Kovacs' reagent). The fermentative abilities of this bacterium are limited; acid is produced from dextrose, but most other carbohydrates are not attacked. An alkaline reaction is produced in litmus milk; a soft coagulum is formed, followed by rapid peptonization, and reduction of the indicator.

One of the most distinctive characteristics of Ps. aeruginosa is its production of a bluish green, soluble pigment which does not color the colonies or other masses of growth but instead diffuses into the medium. There are actually two pigments formed. The one, pyocyanin, is a deep blue in color and can be extracted from aqueous solution by chloroform, and the other, a yellowish green fluorescent pigment, is soluble in water but not in chloroform. They are both oxidation products of colorless precursors. Pyocyanin is formed only by Ps. aeruginosa, but the fluorescent pigment is formed by several other species of Pseudomonas.28 These pigments may occur separately or together. Pyocyanin is of interest in that it is a phenazine.

The serological character of Pseudomonas, especially *Ps. aeruginosa*, was open to question for many years. It is now established that there is antigenic diversity within the species with respect to both H and O antigens, allowing the differentiation of 10 O groups, which in combination with the 10 H antigens, has allowed the separation of 29 agglutinative serotypes. There appears to be no species-specific antigen, but the O antigens of *Ps. aeruginosa* seem not to occur in other Pseudomonas species. Strains

may be separated into a number of phage types, 49,54 but such typing has had little practical application as yet.

Pathogenicity. For some time after its discovery Ps. aeruginosa was generally regarded as a harmless saprophyte, or at the most as a microorganism of slight pathogenic power. It subsequently became clear that this bacterium is causally associated with a great variety of suppurative and other infections of man. It is found in pure culture in abscesses in different parts of the body. especially in the middle ear. 60 Cases of endocarditis,40 pneumonia, and meningitis,47 though rare, also occur in which Ps. aeruginosa appears to be the sole responsible microorganism. A generalized and fatal form of pyocyanic infection occurs, and the bacillus has been found in the blood during life.8 Ps. aeruginosa has beeen associated, possibly etiologically, with waterborne diarrheal disease.30

Infections with *Ps. aeruginosa* and other Pseudomonas species are difficult to treat because they do not respond well or uniformly to antibacterial drugs. In general, polymyxin is the most consistently active, streptomycin and the tetracyclines are less active and give variable results, and penicillin, erythromycin, and other antibiotics affecting the gram-positive bacteria have substantially no therapeutic effect.²

The intraperitoneal injection of 0.25 ml. of culture of a virulent strain will kill a guinea pig with acute symptoms in 24 hours. Smaller amounts are also fatal but less rapidly. Subcutaneous inoculation produces a marked local reaction. The symptom complex presents nothing especially characteristic. Rabbits are not so susceptible as guinea pigs; mice and pigeons are less susceptible than rabbits. Immunity can be produced by small, nonlethal doses.

Lactobacillus

Microorganisms comprising this somewhat loosely assembled genus produce considerable amounts of lactic acid from the simpler carbohydrates and concomitantly withstand a degree of acidity usually fatal to nonsporulating bacteria. This acid-tolerant attribute of these bacteria, which are termed aciduric, is extremely useful in the isolation of cultures as well as in distinguishing the group.

Morphologically some of the lactobacilli are long, slender rods, while others are somewhat similar to the colon bacillus, but, unlike it, they are all gram-positive. Almost all are nonmotile, but exceptions have been reported. Many cultures exhibit a typical diplobacillary form, often kidney-shaped. Old cultures frequently show considerable pleomorphism.

Physiology. The lactobacilli are microaerophilic or anaerobic on primary isolation. but after continued cultivation some strains will grow in the presence of air. Their nutritional requirements are complex, and most strains cannot be cultivated on the usual nutrient or infusion mediums unless these are enriched by the addition of glucose or whey. Individual requirements for amino acids vary from two to 15, and pyridoxine, thiamin, riboflavin, biotin, folic acid, and nicotinic acid are required in general, individual requirements differing. Such varied nutritional requirements have valuable practical application in microbiological assay procedures for vitamins and certain amino acids, for they are more sensitive than available chemical methods. Within an appropriate concentration range, there is a definite, even linear, relation between the concentration of vitamin in a vitamin-free but otherwise nutritionally adequate culture medium and the amount of growth or acid produced.

Some lactobacilli are a part of the normal intestinal flora and may predominate in infants and others having a high intake of sugars, especially lactose. At one time it was supposed that a lactobacillus intestinal flora was preferable to a coliform proteolytic flora because it tended to inhibit degenerative changes with increased vitality in the higher age groups, and that such a flora could be established by consumption of fermented milks, acidophilus and bulgaricus milks. The composition of the intestinal flora may be altered in this way, and also by the consumption of equal amounts of sweet milk, but the change is temporary, and in any case there is no evidence that such an intestinal flora in itself favors general health.

While rare instances of association of lactobacilli with pathological conditions, such as endocarditis and febrile disease, have been noted, these bacteria are essentially nonpathogenic except insofar as they may be associated with dental caries (see below). They are primarily of interest in the dairy and fermentation industries, where they are of considerable importance.

Classification. The classification of lactobacilli has been based on the source from which they were isolated. Such a basis is unsatisfactory except possibly in the case of certain obligate parasitic bacteria having highly pronounced host specificities. No other means of differentiation and characterization has been completely satisfactory and, although physiological properties vary widely from strain to strain, at the present time species of lactobacilli are separated on a physiological basis, using optimum growth temperatures, anaerobiasis, and certain sugar fermentations.

The lactobacilli fall into two groups on the basis of the products of sugar fermentation. The homofermentative group, which is the largest, converts the fermented sugar almost entirely to lactic acid, while the heterofermentative group is made up of the forms which produce considerable amounts of other products of fermentation, including carbon dioxide, ethanol, and acetic acid. According to the Bergey classification, these two groups are divided into species in the following way:

Homofermentative lactobacilli:

(A) having an optimum temperature of 37° to 60° C. or higher

(1) produce acid from lactose

- (a) having an optimum temperature of 37° to 45° C.
 - (i) produce l-lactic acid Lactobacillus caucasicus Lactobacillus lactis
 - (ii) produce dl- or d-lactic acid micro-aerophilic Lactobacillus helveticus Lactobacillus acidophilus
 - (iii) anaerobic in freshly isolated cultures Lactobacillus bifidus
- (b) having an optimum temperature of 45° to 62° C.

Lactobacillus bulgaricus Lactobacillus thermophilus

- (2) do not produce acid from lactose Lactobacillus delbrueckii
- (B) having an optimum temperature between 28° and
 - (1) produce optically active lactic acid (a) produce d-lactic acid

Lactobacillus casei (b) produce l-lactic acid

Lactobacillus leichmannii

(2) produce optically inactive lactic acid Lactobacillus plantarum

Heterofermentative lactobacilli:

(A) having an optimum temperature of 28° to 32° C. (1) ferment raffinose, sucrose, and lactose

Lactobacillus pastorianus Lactobacillus buchneri

(2) do not ferment raffinose, and often not sucrose or lactose

Lactobacillus brevis

(B) having an optimum temperature of 35° to 40° C. or higher

Lactobacillus fermenti

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A number of studies of the serological relationships among the lactobacilli have been



Figure 144. Lactobacillus sp. isolated from the mouth. Morphologically identical with L. acidophilus. Note the diplobacillary form and palisade arrangement of the cell. × 2500. (Harrison.)

carried out, using polysaccharides extracted from the bacteria as antigens, and the conventional agglutinin-agglutinin absorption methods. In the first instance, four serological types were separated but appeared to be somewhat unstable, changes in antigenicity being associated with changes in fermentation reactions.^{24, 25, 26} While the study of agglutinogens is complicated by the marked tendency of lactobacilli to spontaneous agglutination in salt solution, 10 different agglutinogens are demonstrable among the homofermentative lactobacilli, together with certain minor antigens shared with Leuconostoc species.^{44, 74, 75}

Of the lactobacillus species distinguished by physiological reactions, only three need be considered here.

by Moro in 1900 from the feces of infants, this organism has been isolated from the intestine of nearly all the mammalia, many other vertebrates, and some of the invertebrates. Its number increases in the intestine when the carbohydrate content of the diet is increased and may become predominant when a milk diet is ingested. These bacilli, fairly large and of variable length, are arranged singly, in pairs frequently slightly bent at the juncture, and in palisades. Long chains, filamentous forms, and club shapes are not uncommon. Young cultures stain

uniformly gram-positive; old cultures often show beading or bipolar staining and may be easily destained. Colonies, usually small, may vary in shape between a smooth, rounded. opaque form and a flattened, translucent, irregular form often having a ground-glass appearance. Fermentation reactions are variable, but most strains produce acid (no gas) from glucose, lactose, maltose, and sucrose and coagulate milk within 48 hours. Döderlein's bacillus (1892), a common constituent of the flora of the vagina and believed to aid in the natural defenses against infection by contributing to the acidity of the vaginal secretions, is considered to be identical with L. acidophilus.

Lactobacillus bifidus. Apparently closely related to L. acidophilus and often difficult to distinguish from it, L. bifidus is a thinner rod with ends somewhat more tapering and usually bifurcated on isolation. It was isolated from feces of breast-fed infants by Tissier in 1900. Although common in the intestine of breast-fed infants, sometimes comprising over 90 per cent of the total intestinal flora, it is less conspicuous in the intestinal contents of bottle-fed babies. It is sometimes found also in the feces of adult animals, including man. Like L. acidophilus it produces acid, mainly lactic, from a number of sugars but also ferments inulin. It is anaerobic on primary isolation, and some strains never grow well under aerobic conditions. Growth is enhanced by cystine. Partly because of its anaerobic requirements it has been classified with the Bacteroides.

Lactobacillus bulgaricus. This name was assigned to an organism isolated by Grigoroff in 1905 from Bulgarian fermented milk. It gained prominence through the work of Metchnikoff, who believed that intestinal putrefaction could be restrained by drinking milk fermented by this organism as noted above. When it was later shown that L. bulgaricus does not become implanted in the intestine, its use in experimental therapeusis was dropped in favor of L. acidophilus. More difficult to cultivate than L. acidophilus, slightly larger, and somewhat different in sugar fermentations, it is nevertheless closely related. It has been reported that L. bulgaricus rarely grows at 15° C., dies out on repeated culture in a lactose-peptoneyeast extract broth, is unable to grow in mediums containing 2.5 per cent sodium chloride, and fails to grow in broth at pH

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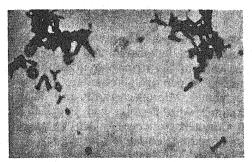


Figure 145. Lactobacillus bifidus. Note the Y-shaped forms. (Dack.)

7.8, while *L. acidophilus* will grow under all these conditions. The Boas-Oppler bacillus, first seen in 1895 in the gastric juice of patients suffering from stomach carcinoma, is a member of this group, similar to if not identical with *L. bulgaricus*.

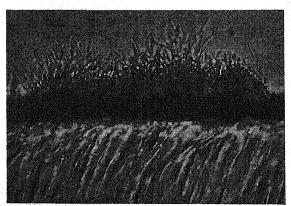
Dental caries. 66 Decalcification of the tooth, which is a prominent part of dental caries, may be brought about by organic acids of microbial origin. Acid formation occurs rapidly in dental plaques (see below) as measured by microelectrodes following oral rinse with glucose solution, reaching concentrations sufficient for decalcification in vitro. The lactobacilli are not only constantly present in the mouth and induce a rapid conversion of carbohydrate to lactic acid, but their aciduric nature allows their persistence in such acidities. It has been sus-

pected, therefore, that they may be etiologically related to the carious process.

The mouth contains a wide variety of bacteria, such as streptococci, filamentous forms, bacteroides, and spirochetes, in addition to lactobacilli. The composition of the flora is affected by the nature of the diet, including dietary deficiencies, and by other factors such as antibiotic therapy, which often results in a predominantly coliform flora. It is also significantly altered when certain pathological conditions are present in the mouth, and such alterations may reflect any part played, particularly by those forms which increase in number, in the pathological process.

In general, gingivitis and periodontal disease are associated with a predominantly proteolytic flora. Involvement of the gum margins may range from mild involvement of the buccal and lingual margins and interdental papillae to the acute or chronic ulceromembranous gingivitis known as Vincent's disease. The classic microscopic picture of spirochetes and fusiform bacilli diagnostic of Vincent's angina (Chap. Thirty-three) is incomplete in that a variety of other forms may be found in the masses of bacteria growing along the gingival margin.

The carious lesion is commonly initiated beneath a tooth plaque, a felt-like mass on the surface of the tooth made up of filamentous microorganisms in which various kinds of bacteria are entrapped. The predominant forms are α -hemolytic streptococci, gram-



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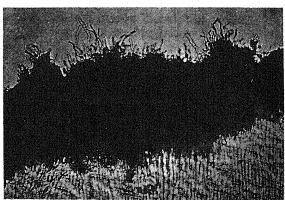


Figure 146. Left, ground section of human tooth enamel showing an unstained bacterial plaque growing on the surface. Note the enamel rods and filamentous bacteria. × 1200. Right, ground section through an early carious lesion in human tooth enamel stained with gentian violet. Note the intimate contact of the bacterial plaque with the eroding surface. × 500. (Blayney.)

594

positive diplococci, diphtheroid bacilli, and anaerobic fusiform bacilli, together with smaller numbers of lactobacilli. Neisseria. and other gram-negative cocci. It has been found generally, though not invariably, that the number of lactobacilli present in the saliva increases during active caries, and that both the development of the carious process and the increase in number of lactobacilli are arrested by sharp restriction of sugars in the diet. Such observations have been taken to suggest a role of the lactobacillus flora in the decalcification of caries, and there would seem to be little doubt that bacteria are causally involved, since experimental caries does not occur in germ-free animals.45

More recent evidence, based on studies with germ-free animals and animals in which portions of the bacterial flora have been depressed with antibiotics, have cast doubt on a role being played by the lactobacilli in the initiation of the carious process: these studies have tended to implicate certain kinds, or strains, of streptococci.31 It will be recalled that the streptococci, like the lactobacilli, are distinguished by the fermentation of glucose primarily to lactic acid, and the Streptococceae and Lactobacilleae are the two tribes making up the family Lactobacillaceae. In addition, the streptococci are also proteolytic, and proteolysis is involved also in the development of caries. Whether there is a specific etiology of dental caries, or whether the carious process results from the activity of more than one kind of bacteria as seems to be the case in periodontal disease, remains to be established, but it seems probable that certain streptococci, and possibly also lactobacilli because of their aciduric nature, are significant elements.

RELATED BACTERIA

Microbacterium. Forms which are closely related to the lactobacilli and produce lactic acid without gas in carbohydrate fermentation, but which are aerobic, are grouped in the genus Microbacterium and formally classified with Listeria and Erysipelothrix as Corynebacteriaceae. Two species are recognized, Microbacterium lacticum and M. flavum. The first occurs in the intestinal tract, and the second predominantly in dairy products. Their relatively high heat resistance is of significance in the latter.

Propionic acid bacteria. A group of bacteria closely related to the lactobacilli, but characterized by the production of propionic acid and acetic acid in the fermentation of carbohydrates and polyhydric alcohols, is known as the propionic acid bacteria. The original single species, Bacterium acidi propionici, has been split into a number of species on a physiological basis under the generic name Propionibacterium. Of the 11 species recognized by Bergey all but one are anaerobic or micro-aerophilic.

They are nonmotile, nonspore-forming, gram-positive bacilli somewhat resembling the diphtheroids in microscopic morphology in that they contain metachromatic granules, and the cells are often club-shaped and show branching. Growth is relatively slow, and their nutritional requirements are complex in that yeast extract mediums supplemented with lactate or monosaccharides are required to support growth. These bacteria are of some industrial importance and have been widely used in studies on the mechanisms of bacterial fermentation of sugars. They seem to be completely nonpathogenic.

Listeria monocytogenes^{22, 61}

The microorganism now known as Listeria monocytogenes has undoubtedly been isolated from diseased lower animals and man prior to 1926, but without sufficiently precise characterization to allow its identification. In that year it was described by Murray, Webb, and Swann as the etiologic agent of an epizootic among laboratory rabbits and guinea pigs which was characterized in part by a monocytosis, and it was named by them Bacterium monocytogenes. What proved to be the same microorganism was isolated the following year by Pirie from the gerbil

(Tatera lobengulae) in South Africa, and named by him Listerella hepatolytica because of the lesions produced in the liver in experimentally infected animals. When the identity of these isolates was established, the microorganism was given the name Listerella monocytogenes. Following the objection of systematists that the generic name Listerella had been pre-empted, the name was changed to Listeria monocytogenes, by which it continues to be known despite further objections by systematists that this generic name had also been pre-empted. It

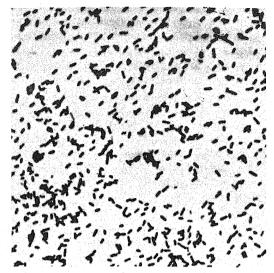


Figure 147. Listeria monocytogenes. Smear from a pure culture. Fuchsin: × 1050.

has been isolated since, but not recognized owing to insufficiently critical characterization, and the names Corynebacterium infantisepticum and Corynebacterium parvulum occur in the literature.

Morphology and staining. This microorganism is a nonsporulating, nonencapsulated rod with slightly pointed ends. In very young cultures it is found in bacillary form, but later it is predominantly coccoid, $0.5 \mu \times 1$ to 2μ , the form in which it is usually observed. In old cultures or cultures of the rough form, filaments 6 to 20 μ in length occur. The arrangement of the cells is not characteristic; they may be found singly, in pairs sometimes at an acute angle to one another, and in short chains.

In young cultures the bacilli are invariably gram-positive, but as the culture ages the Gram reaction becomes irregular. On partial decolorization as observed in older cultures, the center portion of the cell is decolorized first to suggest bipolar staining, but metachromatic granules and the banded appearance characteristic of the corvnebacteria does not occur. Some of the conventional simple stains, e.g., methylene blue, are not satisfactory, and the Gram stain or Giemsa should be used, especially for tissue sections.

These bacteria are motile, a characteristic which, among others, distinguishes them from Erysipelothrix (with which they are classified by British workers as Erysipelothrix monocytogenes), and motility is demonstrable by swarming in soft agar culture at 20° C. as well as by direct observation. When grown at room temperature there are four or less peritrichous flagella, but when cultured at 37° C. usually only a single polar flagellum is demonstrable.

Growth is diffuse in liquid media, except in the case of rough strains, and on solid media, e.g., tryptose, blood, or serum agar, minute opaque colonies appear in 24 to 48 hours, becoming larger with raised edges on continued incubation. There is a very small zone of \(\beta\)-hemolysis; occasional strains show much greater hemolytic activity on sheep blood agar which is correlated with lecithinase activity of the strain. Two main types of variants are found, the one more translucent and less hemolytic which is agglutinable in saline but retains antigenicity, and the other typically rough.

Physiology. These bacteria are grown, especially on primary isolation, on relatively rich mediums and under increased CO₂ tension. Growth occurs at neutral or slightly alkaline reactions, and these bacteria are unusual in their ability to grow well at pH's as high as 9.6 and in the presence of 10 per cent, or even more, NaCl. Growth is relatively slow in liquid mediums in the absence of fermentable carbohydrate, and may be delayed for one to two weeks or not occur at all when the inoculum is small; this may account for some failures to isolate Listeria from pathological material. When fermentable carbohydrate, e.g., glucose, is included in the medium, growth is much more rapid but, as the pH falls to 5.6 or less, the bacteria die out in two to three days. Enrichment occurs when the specimen is allowed to stand for one to three months in the refrigerator, owing presumably to the ability of these bacteria to grow slowly at such low temperatures, and subsequent subculture may be successful when immediate culture was not. They may be grown on defined mediums containing a number of amino acids.15

These bacteria are relatively inactive biochemically; i.e., they are not proteolytic, do not produce indole or H₂S, and fail to hydrolyze urea or reduce nitrates. Dextrose, maltose, levulose, salicin, and trehalose are almost invariably fermented but without gas formation; sucrose, lactose, glycerol, xylose, and rhamnose are fermented irregularly or only by some strains; and mannitol, dulcitol, arabinose, inositol, and raffinose are not fermented. The separation

596

LISTERIA

of Listeria into fermentative types has not been useful.

With the exception of the enhanced hemolytic activity associated with lecithinase and the irregular fermentation of some carbohydrates, *List. monocytogenes* is remarkably uniform regardless of source. It is generally agreed, therefore, that there is but one species, and various names arising from presumed host specificity, such as *List. ovis, List. cuniculi, List. bovina, List. gerbilli,* and *List. gallinarum,* have no standing.

Antigenic structure.3 Earlier studies on the antigenic specificity of List. monocytogenes indicated that these microorganisms fall into two general serological types, types 1 and 4 below. One of these appeared to occur in rodents, the other in ruminants, and both in man. More precise studies, based on detailed antigenic analysis, have shown that both heat-labile flagellar and heat-stable somatic antigens are present. These allow differentiation of four main serotypes, designated 1, 2, 3, and 4, and the subdivision into subtypes of which type 4a and type 4b appear to be the most important. The antigenic structure of these serotypes is shown in the accompanying table. While List. monocytogenes may be identified and typed by the use of appropriate antiserums, crossreactions have been found to occur with a number of other bacteria, including strains of Str. fecalis, Staph. aureus, E. coli, and various Corynebacterium species. The serodiagnosis of Listeria infection using paired

Antigenic Structure of Listeria monocytogenes*

SEROTYPE	ANTIGENS				
	O (HEAT STABLE)	H (HEAT LABILE)			
la lb	I, II, (III) I, II, (III)	A, B A, B, C			
2	1, 11, (111)	B, D			
3a 3b	H, (IH), IV H, (IH), IV	A, B A, B, C			
4a 4b 4ab 4c 4d 4d	(III), (V), VII, IX (III), V, VI (III), V, VI, VII, IX (III), V, VII (III), VI, VIII (III), V, VI, VIII, IX				

^{*}Modified from Gray and Killinger.

serums from patients is, then, not reliable, for rise in titer to Listeria antigen may occur in infections with other microorganisms.

Toxins. The characteristic monocytosis, to as high as 30 per cent of the total white cell count by the fourth day of experimental infection in rabbits, is doubtless due, at least in large part, to the presence of a monocytosis-producing agent in the microorganisms. This is apparently a lipid substance, liberated by mechanical disruption of the cells and extractable with organic solvents such as chloroform, ether, or petroleum ether. The activity is correlated with virulence, for it is present in large amount in freshly isolated virulent strains, decreases rapidly coincident with decline in virulence on serial culture on laboratory mediums, and may be restored by animal passage. The partially purified material produces an effect on the hematopoietic system substantially identical with that which occurs in experimental infection, but it does not appear to be otherwise toxic. Whether polysaccharide fractions of the bacilli are toxic as has been reported has not been established. A protein substance, separable from mechanically disrupted cells and precipitable by ammonium sulfate, has been reported to have a virulence-enhancing effect.48 These bacteria appear to have no endotoxin in the conventional sense.

Pathogenicity. Naturally occurring infection of lower animals and man, or lisapparently world-wide teriosis. is distribution though most of the reported cases have been in this country and Western Europe. There have been several thousand confirmed infections in lower animals, particularly domestic animals, and more than 700 confirmed human cases have been recorded. It is probable that many are missed because the disease cannot be diagnosed with certainty on clinical grounds alone, and because of failure to isolate and/or recognize the microorganism.

In lower animals the disease occurs in both sporadic and epizootic form. It assumes a generalized septicemic form in rodents and in young animals generally, with the development of necrotic foci in the liver. In older animals there is a tendency to involvement of the central nervous system with resulting encephalitis or encephalomyelitis. The infection also has a tendency to localize in the reproductive system, resulting in abortion in pregnant animals. Among domes-

tic animals the sheep appears to be among the most susceptible, and the disease takes a fulminating form with high mortality, at times assuming considerable economic importance. The disease is known as "circling disease" in these animals, but relevant symptoms of central nervous system infection are not invariably present. Cattle are infected also, and swine less often; the horse is infected only occasionally. Epizootics have occurred in colonies of furbearing animals such as foxes and chinchillas.

Of the common experimental animals³³, the mouse and rabbit appear to be among the most susceptible, and fatal infection is readily produced by intravenous or intraperitoneal inoculation. The guinea pig is less susceptible and cannot be fatally infected with regularity. Freshly isolated smooth strains of *List. monocytogenes* appear to be almost invariably highly virulent, and test for virulence is a significant element in its characterization, but, since virulence is lost readily on culture on laboratory mediums, it may appear to differ widely from strain to strain when culture strains are tested.

Listeriosis in man may be a localized infection, cutaneous or of mucous membranes of varying severity to give upper respiratory symptoms, angina, involvement of regional lymphatics, and at times associated conjunctivitis. Monocytosis is common in this kind of infection. Or it may assume, or progress to, a generalized septicemic form with infection of the reproductive system and transplacental transmission to the fetus in pregnant women, or neonatal infection or involve the central nervous system with resulting encephalitis. Histologically the lesions are granulomas and focal necroses. Diagnosis cannot be based on symptomatology; the septic form with angina and monocytosis is indistinguishable from infectious mononucleosis of viral or other etiology (Chap. Thirty-seven), the encephalitis, in which monocytosis is not a constant feature, does not differ significantly from encephalitis of other bacterial or viral etiology, etc. The case fatality rate appears to be high, perhaps 70 per cent when the central nervous system is involved. At the same time, asymptomatic and abortive infections occur, but their relative frequency is not known. Tetracyclines and sulfonamides are considered to be reasonably effective chemotherapeutic agents if given early; penicillin is apparently not consistently effective. The microorganism may be isolated from the blood, or bone marrow by sternal puncture, early in the disease; from purulent material taken from lymph nodes and cutaneous lesions; from swab specimens of infected mucous membranes; and from spinal fluid when the central nervous system is infected.

Epidemiology.²¹ The epidemiology listeriosis is as yet far from clear. It is considered to be primarily a disease of lower animals, possibly with a reservoir of infection in rodents as suggested by the isolation of the microorganisms from such animals in the wild state. Nevertheless, it has been possible in only relatively few instances of confirmed infection to trace the source of infection. Among domestic animals an association with certain feeds, especially silage, has been noted by many workers, and List. monocytogenes has, in fact, been isolated directly from silage. The source of contamination of silage is another matter, and a saprophytic stage in the life history of the microorganism cannot be ruled out. It is probable that human infection is acquired from lower animals through direct or indirect contact. With the exception of transplacental infection of the human fetus, or neonatal infection acquired at birth, the disease seems not to be transmitted from man to man.

Erysipelothrix rhusiopathiae 76

Microorganisms closely related to the actinomycetes have been found to be the causative agents of swine erysipelas and a type of mouse septicemia; they also infect man, and the disease produced is termed erysipeloid to distinguish it from erysipelas of streptococcal etiology. It was thought

for a time that the mouse septicemia organism isolated by Koch, the organism isolated from swine by Pasteur and Thuillier and by Löffler, and that found by Rosenbach in human erysipeloid were distinct species of a genus to which the name Erysipelothrix has been given, and they were called Ery-

sipelothrix muriseptica, Ery. rhusiopathiae, and Ery. erysipeloides respectively. The first of these, sometimes called Bact, murisepticum, is not to be confused with Pasteurella muriseptica, which also has the same synonym. Other names that have been used are Ery. rhusiopathiae suis, Ery. porci, erysipelatis suis, Actinomyces Bacillus rhusiopathiae, and the swine rotlauf bacillus. It is now generally agreed that these organisms are identical, or at least closely related varieties of the same species, for while their morphology is variable they are immunologically identical and are recognized as a single species, Ery. rhusiopathiae. These microorganisms are regarded by some workers as related to L. monocytogenes.

Morphology and physiology.64 Ery. rhusiopathiae occurs in two rather well defined morphological types usually designated smooth and rough though their relationship to the S and R variants of bacterial dissociation is not clear. The smooth type appears as a small, slender, sometimes slightly curved, nonmotile, nonsporulating, grampositive rod. Long chains of bacilli and filaments, sometimes beaded and showing swollen areas, are present in smears of the rough form. Both stain readily and sometimes irregularly with deeply staining granules. Colonies of the smooth form on solid mediums are round, convex, amorphous, water-clear, and small, perhaps 0.1 mm. in diameter, and broth cultures are uniformly turbid. The rough form produces larger colonies with a granular, curled appearance like that of very small colonies of anthrax bacilli, while growth in liquid mediums is in the form of flocculent, hair-like masses with little or no turbidity. The most characteristic growth is in gelatin stab cultures; bead-like colonies appear along the line of inoculation from which lateral filamentous growth occurs, resembling a test-tube brush.

The organism is micro-aerophilic but will grow under aerobic or anaerobic conditions; growth appears on the usual infusion mediums with 24 hours' incubation at the optimum temperature of 30° C. There is no growth on potato. Fermentation reactions are variable from strain to strain, but most ferment dextrose, lactose, and levulose. Nitrate is reduced and hydrogen sulfide is produced, but indol is not formed, and litmus milk is unchanged or slightly acidified.

Ery. rhusiopathiae is somewhat more

than ordinarily resistant to drying and to various preservative processes such as smoking, pickling, and salting and survives for relatively long periods in putrefying meat and in water. It is probably in part because of survival of the organisms in filth that infected areas experience recurrences of the disease year after year.

Pathogenicity. In swine, four clinical types of disease are found. In the acute septicemic form, with lesions in the viscera and internal organs, the case fatality rate is high, perhaps 80 per cent, with death in three to five days. The urticarial form, known as "diamonds" or "diamond skin disease" because of the occurrence of reddish to purplish rhomboidal blotches on the skin, may occur with or without visceral involvement and is seldom fatal. The chronic form is a vegetative endocarditis with erosion of the mitral valves in particular; death always results eventually. Arthritis may complicate the other clinical types or occur independently; it is usually not fatal, but growth is stunted. The organisms are excreted in large numbers in the feces, and it is generally assumed that natural infection takes place by mouth although experimental feeding has given irregular results. Infection is spread in part by healthy carriers as well as diseased animals, and pork trimmings in garbage probably account for isolated cases. Swine erysipelas is of very great economic importance in Europe, especially in Germany where it is known as Schweinerotlauf, and in recent years has been found to be



Figure 148. Erysipelothrix rhusiopathiae, pure culture. Note the similarity of this microorganism to the actinomycetes. × 1000. (Kral.)

LISTERIOSIS 599

more important in this country, at least in certain areas, than formerly thought.

Sheep are occasionally infected with Ery. rhusiopathiae and develop a polyarthritic form of the disease. The organism is also pathogenic for a variety of birds, and in this country turkeys are most often seriously affected; cyanosis is a prominent clinical feature and evident in the "blue comb." It has also been found in wild rats, which should, perhaps, be considered as a reservoir of infection and possibly a source of the human disease.

The disease may be reproduced by the inoculation of swine, though the results are irregular. Of the usual laboratory animals, white mice and pigeons are highly susceptible, dying in one to four days after subcutaneous inoculation, and are commonly used in diagnosis. The rabbit is not very susceptible, and the guinea pig is quite resistant. In general, the virulence of the microorganism varies widely.

Pathogenicity for man.34,56 Human infection with Ery. rhusiopathiae is well known. The septicemic type with diffuse erythema is rare in man, and only a very few instances have been reported. The chronic form with endocarditis and polyarthritis is also very rare. The usual type of infection is an erythematous-edematous lesion, the local lesion commonly developing on the fingers or hand from an abrasion where the infection enters. The lesion, although spreading, never extends beyond the wrist. There is some swelling and a marked erythema of the lesion and sometimes local arthritis and regional adenitis. The disease is usually self-limiting and terminates within a month. The organism may be cultivated from an excised piece of skin from the lesion.

Human infection can almost invariably be traced to contact with animals and animal products such as meat, hides, bones, and manure, or to fish and shellfish, and the disease is, therefore, associated with certain occupations.³⁵ For example, more than half the cases found in the Philadelphia

region were in slaughterhouse workers. The disease has been observed in workers in a bone button factory using cattle and hog bones, and it not uncommon in fishermen and fish handlers. It also occurs in persons working in kitchens with raw meat and fish. In this country, contact with live fish and crustacea appears to be the chief source of infection. Hettche has reported finding Ery. rhusiopathiae in 10 of 30 specimens of sewage at Königsberg in Prussia and in five of 52 specimens at Munich; the source apparently was slaughterhouses where infected animals were being killed. Under experimental conditions the organisms not only survived for some days in water, but fish developed a latent infection when fed infected meat; the microorganism could be isolated from most organs, including the kidney, and were excreted in large numbers in the urine, contaminating the water.

Immunity. There is a definite immune response in infected hogs as evidenced by the formation of agglutinins which have diagnostic value. Ery. rhusiopathiae is immunologically relatively homogeneous, but serological groups may be distinguished^{32,72} by reciprocal absorption. Antiserum has therapeutic value, and in man is given both intramuscularly and by infiltration about the local lesion. Passive immunization of swine is an effective prophylactic but the immunity lasts no longer than two weeks. Animals may be actively immunized with the vaccine developed by Pasteur and Thuillier, who attenuated the organism by passing it through rabbits. Vaccine erysipelas occurs with some frequency, however, and the method has now been largely superseded by the simultaneous inoculation of virulent culture and antiserum. Since the vaccine maintains and spreads infection in herds, the Bureau of Animal Industry has permitted only its limited use in this country and only in areas where the disease has become prevalent. Both methods provide adequate protection for eight to 12 months; periodic reimmunization is required for breeding stock.

Nonspore-forming Anaerobic Bacilli (Bacteroides)

There is a large group of nonspore-forming anaerobic bacilli that are usually gramnegative. Normal inhabitants of the upper respiratory tract, genital tract, and colon, where they may outnumber the aerobic flora, 78 the microorganisms are not infre-

quently associated with ulcerative processes of the mucous membranes and may, under appropriate circumstances, invade the tissues and organs of the body with the production of abscesses, or the blood stream to give rise to septicemia.^{4, 14, 68} Generally

neglected in routine bacteriological examinations, these bacteria may be present in "sterile pus" from surgically drained abscesses and similar affections in which bacteria are not found by the usual cultural methods. Dack⁹ noted their presence in 200 of 5180 specimens from the Department of Surgery of the University of Chicago submitted for routine bacteriological examination, an incidence of about 4 per cent.

The relation of these microorganisms to other bacteria is uncertain. As a group they probably make up several genera and cannot be regarded as species of a single genus except tentatively. They are morphologically heterogeneous, varying from slender rod forms which may be tapered toward the ends, the so-called fusiform bacilli, to filamentous and branching forms characteristic of the higher fungi. The high degree of pleomorphism characteristic of these forms is due in large part to a mode of reproduction in which large round bodies are formed from which daughter cells separate. These usually resemble the bacillary parent cells but sometimes occur as much smaller elements in the so-called L type of variation. 63

Single species have been given a variety of generic names, including Bacillus, Bacterium, Necrobacillus, Bacterioides, Corynebacterium, Streptothrix, and Actinomyces. Descriptions of these bacteria have been compiled and Prevot⁵⁵ has suggested a more or less elaborate classification.

The Bergey classification separates these forms into two familes and eight genera (exclusive of Streptobacillus) on a morphological basis, viz.:

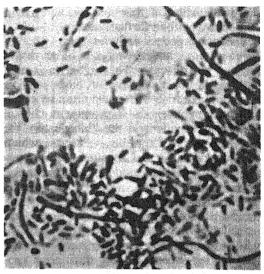


Figure 149. Bacteroides funduliformis. The swollen and filamentous forms and poorly staining "ghost cells" are typical of the usual stained smear preparations. × 1000. (Dack.)

Bacteroidaceae

Bacterioides (rounded ends)

Fusobacterium (pointed ends)

Dialister (minute, diameter of 150 m μ or less)

Sphaerophorus (pleomorphic)

Lactobacillaceae

Lactobacilleae

Eubacterium (no filaments)

Catenabacterium (filaments)

Ramibacterium (false branching)

Cillobacterium (motile)

The difficulty is that the characteristic of failure to form spores and that of requiring anaerobic conditions for growth cover an otherwise heterogeneous group of micro-

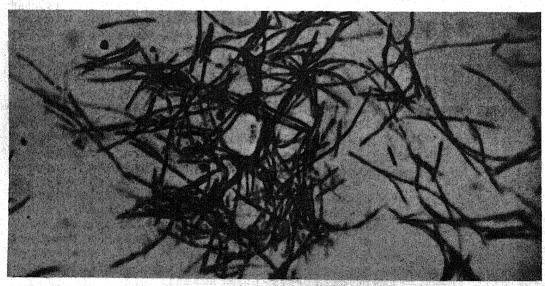


Figure 150. Fusiform bacilli in a stained smear from an anaerobic blood agar culture. × 1800. (Hemmens.)

organisms, not only these but also anaerobic streptococci, Viellonella, etc., most of which are poorly known, both in their relation to one another and to other microorganisms. In view of this, they will, for present purposes, be discussed here under the single name Bacteroides without generic implication in the taxonomic sense.

Those forms which are present in great numbers in normal feces will grow upon the usual laboratory mediums. Those which are associated with pathological processes in man, however, are nutritively fastidious and require infusion mediums enriched by the addition of blood, yeast, or vegetable extracts, and similar substances, together with glucose and cysteine. In some cases they may be isolated on beef or veal infusion blood agar. Some strains grow in amino acid mediums supplemented with all the known bacterial vitamins; pyruvic acid appears to be of considerable importance in the nutrition of these organisms. The optimum pH is 6.3 to 7.0, and the optimum temperature for growth is 37° C. Completely anaerobic conditions are essential, and growth is favored by the presence of carbon dioxide. In vitro they are sensitive to tetracycline and chloramphenicol, but are resistant to streptomycin as well as penicillin.65

The better known of these bacteria may be described briefly.

Bacteroides fusiformis (Bacillus fusiformis, Fusiformis fusiformis, Fusiformis fusiformis, Fusobacterium plauti-vincenti). These fusiform bacilli are found in ulcerative stomatitis (trench mouth) and Vincent's angina. Their relation to the spirochetes with which they are usually associated is considered above and elsewhere (Chap. Thirty-three). They appear as slender rectilinear or incurving

bacilli and frequently assume a filamentous form. They are gram-negative and tend to stain irregularly with the ordinary aniline dyes. Acid but no gas is produced from dextrose, levulose, sucrose, maltose, and sometimes from lactose. They may be isolated on blood agar incubated anaerobically, and the colonies are small and surrounded by a zone of green hemolysis. *Bacteroides fusiformis* is not pathogenic for experimental animals in pure culture, but in mixed culture produces abscesses.

Bacteroides fragilis (Bacillus fragilis, Fusiformis fragilis). This bacterium was found by Veillon and Zuber in 22 cases of appendicitis and since has been found in lung, pelvic, and hepatic abscesses, in septicemia with metastatic abscesses, and in infections of the urinary tract. The cells are small, slender rods, sometimes slightly curved. They are gram-negative and nonmotile. Bact. fragilis is difficult to isolate but will grow on the usual laboratory mediums. The colonies are small (less than 1 mm.) and transparent. No hemolysis is apparent on blood agar. There is a marked tendency to autolysis with apparent resorption of the colonies, and broth cultures are no longer viable after seven to eight days' incubation. A variety of sugars are fermented to acid and gas. The pathogenicity of this bacterium for experimental animals is uncertain.

Bacteroides funduliformis (Actinomyces necrophorus, Fusiformis necrophorus, Streptothrix necrophorus, Bacillus necrophorus, Corynebacterium necrophorum, Schmorl's bacillus). These bacilli have been found in abscesses of the liver, lung, and other parts of the body, in chronic ulcerative colitis, and in the blood stream. Infec-





Figure 151. Bacteroides funduliformis. Electron micrographs. Right, a pair of cells resulting from simple fission. Fixed in formalin; × 3300. Left, swollen cells containing granular material, especially in the swollen areas. Fixed in formalin; × 4900. (Smith, Mudd, and Hillier. 63)

tions with these microorganisms are probably more common than is generally realized. Bact. funduliformis has also been found in lower animals, as in bovine liver abscesses. The bacilli are highly pleomorphic; slender straight and curved rods may be found intermingled with filamentous and swollen forms, and "ghost cells" which do not stain are frequent. There is a marked tendency to irregular staining, and the bacilli are gramnegative. The colonies on blood agar are variable in size from plate to plate and surrounded by a zone of green hemolysis which may become clear upon prolonged exposure to the air. Glucose, maltose, and levulose are fermented to acid, and there is no evidence of proteolytic activity in gelatin or coagulated egg-white cultures. Some strains give rise to a spreading necrotic lesion upon subcutaneous injection in the rabbit, which is usually fatal, while others produce only a localized lesion. Guinea pigs are relatively resistant.

Bacteroides ramosus (Fusiformis mosus, Ramibacterium ramosum, Bacillus ramosus. Not to be confused with Bacillus ramosus as sometimes applied to Bacillus mycoides). These bacteria are frequently present in the pus of appendicitis and have been encountered in pulmonary gangrene. They appear as small, slender rods which often show branching Y forms and pseudofilaments. They are gram-positive. Bact. ramosus is pathogenic for experimental animals, giving rise to abscesses in rabbits and guinea pigs on subcutaneous injection; fatal infections are produced by intravenous inoculation.

Bacteroides melaninogenicus (Ristella melaninogenica). This microorganism has been found in the mouth, tonsils, infected abdominal wounds, focal infections of the

kidneys, in stools from patients with chronic amebic dysentery, and in puerperal sepsis. It is described as a very small gram-negative diplobacillus. On blood agar its colonies are black owing to the slow (four to five days) formation of a melanin-like pigment. Bact. melaninogenicus grows well in mixed culture but sparsely in pure culture. It is difficult to obtain in pure culture and, when admixed with other bacteria in a colony, colors the entire colony black. Acid is formed from dextrose, levulose, lactose, maltose, sucrose, and mannitol. It is markedly proteolytic and rapidly digests coagulated serum and other native proteins. Its pathogenicity as a primary invader is uncertain.

Bacteroides pneumosintes (Dialister pneumosintes). Bact. pneumosintes was described by Olitsky and Gates as the causal agent of influenza. Now known to bear no relation to this disease, its pathogenicity for man is uncertain. It may be cultured from nasopharyngeal washings in Smith-Noguchi medium (human ascitic fluid containing a piece of sterile rabbit kidney and covered with a petrolatum seal) and after a few transfers will grow anaerobically on blood agar, chocolate agar, and Bodet's medium. These bacteria are minute bodies arranged singly. in pairs, or in short chains; they are nonmotile and gram-negative. Because of their small size they pass Berkefeld V and N filters. They produce acid but no gas from dextrose; other sugars are not fermented. If mass cultures are injected intratracheally into rabbits, there is a rise in temperature and sometimes conjunctivitis and mononuclear leucopenia with recovery in two or three days. If the animal is sacrificed, the lungs are found to be edematous with hemorrhages on the surfaces. Bact. pneumosintes is not pathogenic for monkeys.

Streptobacillus moniliformis (Rat-bite Fever, Haverhill Fever)

A microorganism variously known as Streptothrix muris-ratti, Haverhillia multiformis, Streptobacillus moniliformis, Actinomyces muris, and A. muris-ratti is the cause of an acute febrile disease sometimes given the descriptive name erythema multiforme. It is also called rat-bite fever because it may be acquired through the bite of infected rats.

For a good many years the etiology of the disease known as rat-bite fever was con-

fused, for Schottmüller, Blake, and others isolated from the blood of patients a fungus to which the name Streptothrix muris-ratti was given, while Japanese work indicated that a similar disease, known in Japan as sodoku, is caused by a spirochete. In 1925 an epidemic, milkborne disease with unusual and distinctive clinical features occurred in Haverhill, Massachusetts, and was given the descriptive name erythema arthriticum epidemicum or Haverhill fever by Place,

Paritrial instru

Sutton, and Willner.⁵³ The causative microorganism was isolated by Parker and Hudson;⁴⁶ it proved to be a member of the Actinomycetaceae and was given the provisional name *Haverhillia multiformis*. In the same year Levaditi³⁸ independently studied a similar organism from a case clinically like Haverhill fever and named it *Streptobacillus moniliformis*. Other work has yielded similar results. The Japanese work has also been amply confirmed, and it is now clear that there are two types of rat-bite fever, completely different in etiology.

The microorganism is, like the other actinomycetes, pleomorphic in culture with filaments fragmented, branching lary and coccobacillary forms being found in stained smears. Staining may be irregular, and swollen and club-shaped cells are found. It has been suggested that this organism is related to the organisms of the pleuropneumonia group. It is not acid-fast and is gramnegative. It requires enriched mediums for growth; Parker and Hudson found whole rabbit blood, allowed to clot and then inactivated, and broth containing serum or ascitic fluid excellent liquid mediums, and glycerin extract of potato, mixed with infusion broth, enriched with egg yolk, and coagulated by inspissation, the best solid medium. Löffler's serum medium is not particularly satisfactory. Growth is markedly better under anaerobic conditions in the presence of added carbon dioxide than in

When the disease in man is contracted through a rat bite, the initial wound heals, but after a week or 10 days becomes inflamed and painful. Adenitis develops and is followed by toxic symptoms which are the first evidence of the disease acquired from milk. These include headache, chills,

vomiting, and general malaise. An eruption of morbilliform character appears, especially on the extremities, and there is multiple arthritis, often severe. The clinical resemblance to rat-bite fever of spirochetal etiology is very close. The causative microorganism may be isolated by blood culture and has been found in the fluid of affected joints. Agglutinins are formed and according to Brown and Nunemaker⁵ are of considerable diagnostic utility.

S. moniliformis has been found to be a normal parasite of rats and mice and is present in the nasopharynx. It may assume pathogenicity for the rodent host and produce sporadic or epidemic disease of either an acute septicemic or chronic polyarthritic type. An epizootic of the latter type in wild mice has been reported from Australia. The organism has also been found in bronchopneumonia of rats. Although usually of low virulence on artificial inoculation in rats, an experimental infection has been produced with one strain that gives an osteoarthritis.³⁷ It is clear that man may acquire the disease by the bite of normal rats, and probably almost all sporadic cases arise in this way. Brown and Nunemaker are of the opinion that rat bite produces the streptobacillus infection much more commonly than the spirochete infection in this country. The number of undiagnosed cases and the proportion of previously reported cases of rat-bite fever that were S. moniliformis infections are not known. As indicated above, the Haverhill epidemic was milkborne; another milkborne outbreak involving 86 persons was reported in 1934. Parker and Hudson found evidence which suggested that the Haverhill milk was contaminated by an infected cow, but this could not be proved: others have suggested contamination of milk by rats as a possibility.

Bartonella

Bartonella bacilliformis. Oroya fever, an infectious anemia, and verruga peruana, a disease characterized by miliary or nodular eruptions, have existed for centuries in certain districts in Peru and recently have been found in Colombia and Ecuador. It was shown by Carrión, through fatal selfinoculation, that the two are stages or manifestations of a single disease which is now commonly known as Carrión's disease. The etiological agent is a small pleomorphic ba-

cillus which was observed by Barton in 1905 and named *Bartonella bacilliformis* by Strong, Tyzzer, and Sellards.

Morphology and staining.⁵² Bart. bacilliformis is a small, motile, aerobic, gramnegative bacillus 0.2 to 0.5 μ in diameter and 1 to 2 μ in length which is found as a slightly curved rod occurring singly, end to end in pairs, and in short chains. A rounded ovoid form, 0.3 to 1 μ in diameter, is also observed singly, in pairs, and in groups. It

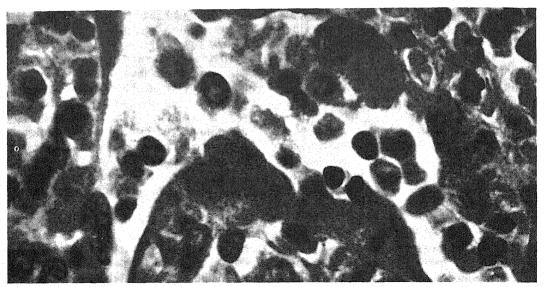


Figure 152. Bartonella bacilliformis in human spleen. Note the huge numbers of the microorganisms packed into the lining cells. Giemsa; × 1450. (Humphreys.)

stains reddish violet with Giemsa's stain, sometimes showing a reddish purple granule at one end of a bluish rod.

Physiology. This microorganism was first cultivated in semisolid leptospira medium and later in tissue culture and in the developing chick embryo, though it could not be carried in serial transfer in the last. Sparse growth occurs on cystine-dextrose-blood agar, and apparently the X factor is required while the V factor is not. Gieman¹⁶ has developed a tryptone-serum medium in both liquid and solid form which supports excellent growth. Little is known of its physiological processes.

Pathogenicity for man. 59 As indicated above, the disease occurs in two forms: the systemic form, in which the red cells are infected; and the histoid cutaneous form. The two may exist independently, coexist, or occur separately, though the usual course is the systemic form followed by the cutaneous form if the former is not fatal, or the cutaneous form alone. The first is a severe. often fatal, febrile anemia. The incubation period is given as about three weeks. The anemia is frequently severe, and the loss of red cells may be 200,000 to 300,000 per cu. mm. per day until the total count is half a million or less. The anemia is thought to be due to the direct action of the microorganisms on the erythrocytes, including hemolysis, and to tissue hemorrhages.⁵⁸ The fever is of an irregular remittent type, and pain in the bones, joints, and head is common. The case fatality rate is 20 to 40 per cent.

death occurring in two to three weeks after onset. With recovery the eruptive stage of the disease appears and persists for two to three months. Whether it follows the systemic form or is the primary clinical manifestation of infection, this stage is characterized by a miliary or nodular eruption; the former is by far the more common. The miliary eruption is most common on the face and extremities, appearing as a macule which becomes nodular and eventually disappears leaving no scar. The nodular type of eruption develops more slowly; the nodules may become 2 to 3 cm. in diameter and have a tendency to become strangulated. They are formed by the proliferation of the endothelial cells of the vessels which become obstructed with an inflammatory exudate of plasma cells and fibroblasts and show a marked tendency to hemorrhage.

Bartonella cells are found in large numbers within the erythrocytes in Orova fever and may be demonstrated in Giemsa-stained blood smears. In both forms of the disease they are found in the tissue macrophages, especially the vascular endothelial cells of the lymphatics, spleen, and liver, often in large clusters within individual cells. The infection is frequently complicated by Salmonella infection to give a high mortality.⁷ It is reported that blood cultures are frequently positive in the systemic infection. but it is not clear whether this is a reliable method of diagnosis. The disease has been treated successfully with chloramphenicol and tetracycline. 50, 69, 77 MYCOPLASMA 605

Bartonellosis is strictly Epidemiology. limited geographically, being, so far as is known, exclusively American and tropical. occurring in the Andes between latitudes 2° N. and 13° S. It is transmitted by Phlebotomus verrucarum and P. noguchii in Peru: whether other species of Phlebotomus or other arthropods, such as Dermacentor. are natural vectors is not known. Peruvian ground squirrels (Citellus tridecemlineatus) have been infected experimentally29 and, together with domestic animals, including chickens, and guinea pigs and field mice, may possibly be naturally infected. Whether there is such an animal reservoir of infection. or the disease is maintained in human infections only, is not known.

Immunity. It is generally said that recovery from an attack of either form of the disease confers a solid immunity to both. With the cultivation of Bart. bacilliformis, it has been possible to study the occurrence of circulating antibody, agglutinins, and investigate the possibilities of prophylactic inoculation. Agglutinins may be demonstrated during the early stages of the disease but, in spite of the lasting immunity, almost always disappear with the subsidence of clinical symptoms. Their diagnostic significance is not as vet known.

Pathogenicity for animals. In general experimental animals appear to be highly resistant to infection with Bart. bacilliformis. Both the eruptive and systemic forms of the disease have been produced in rhesus monkeys, the latter form in splenectomized animals.

Bartonellosis of animals. Naturally occurring bartonellosis has been found in a number of animals, including the dog, cattle, and a variety of rodents. The infection takes a systemic rather than eruptive form and is usually latent, but is activated when host resistance is reduced as by splenectomy. The bartonella-like organisms of lower animals have been separated into three genera: Haemobartonella; Grahamella, containing but a single species (Grahamella talpae) which differs from Haemobartonella in that

the infection is not eradicated by treatment with arsenicals; and Eperythrozoon, which is more highly pleomorphic than the first two groups and is found in the plasma as well as in the erythrocytes. None of these forms has been cultivated, and all appear to be nonpathogenic for man.

Of these, the most commonly encountered is Haemobartonella muris, which infects rats and is transmitted by the rat louse. Haematopinus, and the rat flea, Xenopsylla cheopsis. The infection is very common, and the great majority of laboratory rats are infected. The infection is latent and may be precipitated in an acute form by splenectomy with the appearance of parasitized red cells in the circulating blood. Special strains of rats are maintained bartonella-free for experimental purposes. H. canis is the cause of an infectious anemia of dogs and is transmitted by the dog flea, Ctenocephalus. H. tyzzeri occurs in guinea pigs. H. microtii in the vole, H. bovis in cattle, and other species in mice, shrews, and squirrels.

Eperythrozoonosis similarly occurs as a latent infection which is activated by splenectomy. Eperythrozoon coccoides is a parasite of white mice, and other species occur in wild mice. The disease also occurs in sheep and cattle, and the causative organisms are E. ovis and E. wenyonii respectively.

The systemic position of Bartonella. relationship of these microorganisms to the bacteria on the one hand and to the rickettsiae and viruses on the other is of some interest. Among the bacteria the tendency to parasitize the cells of the host and appear as intracellular clumps of microorganisms is most marked in Past. tularensis, which commonly is found in an intracellular position. Bartonella shows a much more marked preference for intracellular parasitism, and both in this respect and in morphology seems to be closely related to the rickettsiae. It is generally agreed, however, that the relationship is not sufficiently close to justify their classification as rickettsiae, and they may be considered as lying between them and the bacteria.

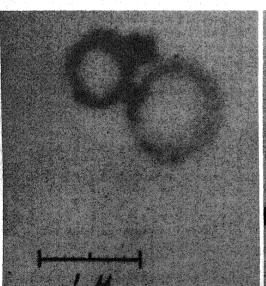
Mycoplasma — The Pleuropneumonia-like Organisms (PPLO)¹²

The first of an apparently more or less homogeneous group of highly pleomorphic, filtrable microorganisms to be described is the causative agent of bovine pleuropneumonia. Subsequently a number of similar forms, both parasitic and saprophytic, have been found. They have been called the pleuropneumonia group or pleuropneumonialike organisms, though none of the rest produces pleuropneumonia in cattle. They are set rather sharply apart from other microorganisms, and are considered to fall into a single genus, Mycoplasma, and the bovine pleuropneumonia organism is taken to be the type species, Mycoplasma mycoides.¹³

Morphology and staining. 11, 71 There are probably few if any other microorganisms which are as highly pleomorphic as these forms. Impression preparations of colonies show granules of various sizes, filaments which may be branched and contain streaming protoplasm, balloon and disc-like structures, ring, club, and star forms, and ameboid structures. Filaments are more numerous in recently isolated cultures and tend not to be formed in older stock cultures. The microscopic morphology of these forms is illustrated in the accompanying figures. The marked pleomorphism is due in part to the fragility of the organisms, many of which are torn apart in making smears, and, as in the case of Bact. funduliformis, in part a consequence of modes of reproduction other than binary fission, e.g., that of the development of round bodies which become nodular with outgrowths that segment into daughter cells. This process has been studied in detail by means of electron micrographs. Viable structures vary greatly in size and include ultramicroscopic elements which are filtrable. Experiments on filtration through gradocol membranes have been described in which the concentration of organisms in an emulsion was reduced from 10^8 to 10^5 by passage through a membrane of APD 0.8 μ . This titer decreased progressively with membranes of decreasing pore size, but complete retention did not occur until a membrane of 0.33 μ was used. In contrast to this, the end point is usually sharp in the filtration of viruses. On the basis of these observations the diameter of the smallest viable elements was estimated to be 165 to 247 m μ . Similar estimates vary somewhat for other strains; the agent of pleuropneumonia, for example, is 125 to 175 m μ .

These forms cannot be found in tissues by any method of staining. They are not demonstrable in smears stained by the usual aniline dyes, but are stained by certain polychrome stains, Giemsa, and Castaneda's rickettsia stain. Very thick films prepared from the sediment of centrifuged cultures are gramnegative.

On primary isolation or change to a slightly different culture medium, broth cultures sometimes, but not always, show no detectable evidence of growth and may be carried along by "blind passage" for several transfers before it appears. Some strains show a uniform opalescence, others a granular type of growth. The characteristic of some strains to grow as small colonies, appearing as flakes attached to the side of the tube, is of some differential value. In any case, visible evidence of growth is very slight, and almost



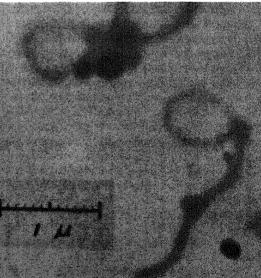


Figure 153. Electron micrographs of the ring forms of the pleuropneumonia-like organisms. (Lilly Research Laboratories.)

all workers with these organisms have found it necessary to carry along an uninoculated tube of medium for comparative purposes.

The colonies upon an agar surface are usually not detectable until after two or three days' incubation. They are most readily observed in stained agar preparations. A square of agar is cut from a suspicious area on the plate and placed on a slide. It is covered with a cover slip on which an alcoholic solution of methylene blue and azure has been dried, and the space between the cover slip and the slide filled with melted paraffin. The average colony size of different strains ranges from 0.01 to 0.6 mm., rarely exceeding the larger figure. The colonies usually have a dark center with a lighter margin and may appear coarsely or finely granular. Upon higher magnification a foamlike structure may be observed which is composed of the balloon-like forms. Oily droplets may be found in the colonies of some strains; these have been found, in some cases at least, to be largely cholesterol already present in the medium. Possibly the pseudocolonies which have been observed on uninoculated serum agar are of similar nature; it is said that these are readily differentiated from true colonies by experienced observers.

Physiology.^{1, 62} These microorganisms require infusion or digest mediums enriched by the addition of serum or ascitic fluid in relatively large amounts. Their growth requirements, especially on primary isolation, have been studied by Morton and his coworkers.^{41, 42} They have shown that these microorganisms are regularly cultivable on a beef heart infusion medium at pH 7.8, containing Bacto peptone or Parke, Davis bac-

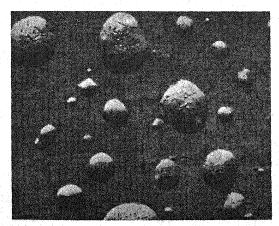


Figure 154. Colonies of a type B pleuropneumonialike organism from mice. Three days' incubation. \times 100. (Sabin: Bacteriol. Rev.)

teriological peptone, and enriched with a heat-stable factor present in ascitic fluid and serum. In some cases these organisms tolerate a relatively wide pH range, but others die out at pH 7.0 and below, and require a pH of 7.8 to 8.0 for growth. The saprophytic varieties will grow at 22° C. with an optimum at 30° C., but the parasitic ones require 37° C. Growth occurs both aerobically and anaerobically but is less abundant in most strains under anaerobic conditions. Fairly heavy inoculums are required, 0.1 to 0.2 ml. of minced tissue in primary isolation, with the transfer of similar amounts of broth cultures, and in transfer from agar cultures a small section of the medium is cut out and dropped into liquid mediums or streaked on another plate. Curiously, their primary isolation is, as a rule, accomplished more readily than their maintenance. These organisms may also be cultured on the chorioallantoic membrane of the developing hen's egg. They are also cultivable in tissue culture, but do not produce a cytopathic effect; their presence in tissue cultures, grossly inapparent, may complicate the use of such substrates for the cultivation of animal viruses.

Some sugars, notably glucose, are fermented, but the pH seldom drops below 7.0 owing to the death of the organisms at this point; the addition of other sugars may inhibit growth. In general, fermentations and other biochemical characteristics have no differential value. The heat resistance of some strains is of the same order as that of most bacteria, while others appear to be more frail and are killed by exposure to 45° C. for 15 minutes. Cultures are best preserved at incubator temperature in mediums containing no added sugar; when sealed with petrolatum the organism may remain viable for a month or more.

Varieties, strains, or species. Strains of these organisms have been isolated from a variety of sources. Those which are parasitic are definitely set off from the saprophytic types, and differentiation or identification within these groups has been made in part on source and pathogenicity and in part on an immunological (agglutination) basis.

With the study of bacterial protoplasts and their similarities to both the L forms of bacteria and the pleuropneumonia-like organisms (PPLO), and the association of the last two with one another (see below), the existence of the PPLO as a group of independent entities becomes uncertain. It has

been urged by a number of workers, especially Dienes, that the PPLO represent stages in the life history of microorganisms better known in other forms. Although there is no convincing evidence that an orderly, cyclical transformation of morphological types occurs among bacteria in this sense (Chap. Seven), at the same time the possibility that the PPLO are naturally occurring protoplasts, differing from the parent microorganism only in lacking a cell wall structure, is highly suggestive. While, then, the PPLO are considered to be independent entities, and given the generic name Mycoplasma in a separate order Mycoplasmatales, any attempt to do other than describe these forms, their origin, and the pathological conditions with which they have been associated seems premature.

Bovine pleuropneumonia. As indicated above, the causative organism of pleuropneumonia of cattle was the first of these forms to be isolated and studied. The disease is found all over the world except in India. Western Europe, and North America; it has been imported into this country a number of times but was finally eradicated by the slaughter of infected animals and has not occurred since 1892.

The natural disease in cattle is characterized by extensive consolidation and subpleural effusion in either or both lungs, and the microorganism is present in large numbers in the serous exudate. It spreads slowly in herds and may take an acute form with death within a week or a chronic form with walling off of the foci of infection. There is occasional joint involvement in young animals. The natural disease has not been reproduced in cattle by either inoculation of infectious serous exudate or of cultures, but an extensive edema develops and spreads from the site of inoculation, and there is a febrile reaction and sometimes death.

The organism is apparently completely nonpathogenic for the usual experimental animals and for man; it has been reported that cultures in sheep- or horse-serum mediums are infectious for sheep and goats, while those in bovine-serum mediums are not.

The name Asterococcus mycoides was given the organism by Borrel and Dujardin-Beaumetz but has not been generally used. In any case, strains obtained in different localities and at different times appear to be very similar if not identical.

Contagious agalactia of sheep and goats. Despite its name this disease is a generalized infection which affects both males and females. It occurs only in parts of southern Europe and North Africa; agalactia or mastitis of these animals in this country is of other etiology.

The lesions occur in the joints and eyes and in the mammary glands of females. The microorganism is present in the blood early in the disease and later may be isolated from the affected regions and from the mammary secretions. The second member of the group to be studied, it was isolated by Bridré and Donatien in 1923. It is morphologically and biochemically very similar to the pleuropneumonia organism and other members of the group but is immunologically distinct and of characteristic pathogenicity. The disease is readily reproduced by inoculation of sheep and goats with cultures.

Canine variety. Organisms of the pleuropneumonia group have also been found in dogs. Shoetensak reported in 1934 the cultivation of an organism of this type from the purulent nasal discharge of dogs ill with distemper which he called Asterococcus canis. In later studies other organisms were found, and there appeared to be two types, immunologically distinct from one another, which he called type I and type II respectively. Their postulated etiological relationship to distemper, generally regarded as a virus disease, is not established.

Varieties from rats. A series of pleuropneumonia-like organisms has been isolated by Klieneberger and her associates from the respiratory tract and elsewhere in normal and diseased rats. Others have isolated similar organisms from rats exhibiting polyarthritis and swollen extremities. These comprise, with a single exception, the "L" series of strains in which the strains are designated by subscript numerals. There now appear to be three distinct types, differentiated on a biological and immunological basis, viz., L_1 , L_3 , and L_4 . The strain L_2 was isolated from a guinea pig and insufficiently studied before loss of the culture at the outbreak of war. The strain described as L₅ is immunologically identical with mouse type A discussed below; L₆ has been insufficiently studied; and L7 has been found to be identical with L₄.

Considerable interest has attached to the apparent association of L₁ with S. moniliformis. Klieneberger-Nobel has been able to isolate L_1 from many strains and stock cultures of S. moniliformis, and it will be recalled that this organism is a natural parasite of the nasopharynx of rats and mice. It is maintained by Klieneberger-Nobel that L_1 and streptobacillus coexist as symbionts, since she has been able to separate the two on the basis of differential resistance to heat and aging, has observed L_1 in the rat in the absence of S. moniliformis, and has carried L_1 strains through many transplants without its reappearance.

These L forms are associated, causally in some instances at least, with relatively mild, chronic affections of rats in which a not uncommon manifestation is joint involvement and polyarthritis. They closely resemble the other organisms of the group morphologically and culturally, forming a subgroup on the basis of pathogenicity, and differentiated from one another immunolog-

ically.

Varieties from mice. Sabin divides the pleuropneumonia-like organisms found in mice into five types, designated as A, B, C, D, and E. Type A is found in normal mice, once in the brain and frequently in the eyes, nasal mucosa, and lungs of carriers. On intracerebral inoculation of mice an ataxia is produced which is characterized by a turning or rolling of the body. The brain lesions and symptoms arising from them are attributable to the action of a soluble toxin produced by the microorganisms.67 immunologically identical organism was isolated by Findlay et al. from mice affected with "rolling disease" and designated L₅. Type B has likewise been found in normal mice and is not only immunologically distinct but produces a progressive arthritis almost exclusively when parenterally inoculated into mice. Types C, D, and E are similar in their pathogenicity but are immunologically distinct; they have not been studied as extensively as some of the others.

Saprophytic varieties. Pleuropneumonialike organisms presumably living a saprophytic existence in nature have been found by Laidlaw and Elford in raw London sewage. They closely resembled the parasitic forms both morphologically and culturally and fell into three immunological groups which were designated types A, B, and C. All were nonpathogenic for experimental animals. Similar forms have been found in Germany in compost and other types of decomposing organic matter.

MYCOPLASMA OF MAN²⁷

Within the past few years interest in Mycoplasma has been greatly stimulated by the demonstration of the etiological relation of one kind of these agents to human pneumonia. A number of species, or strains, differentiable from one another largely, though not entirely, on an immunological basis have been defined. These include M. hominis, types 1 and 2, and M. fermentans, found in the genitourinary tract; M. salivarium and M. orale (M. pharyngis), occurring in the upper respiratory tract; and M. pneumoniae, the causal agent of primary atypical pneumonia. Other varieties have been isolated from the urogenital tract which are as yet poorly characterized, and are known collectively as the T strains.

Primary atypical pneumonia.6 During the 1940's the disease known as primary atypical pneumonia, or PAP, was characterized as a clinical entity, but it became increasingly apparent that this seeming entity may be of diverse etiology. In some instances the causal agent is the psittacosis agent, in others adenoviruses and in still others the disease may be caused by such diverse microorganisms as Histoplasma, Coccioides, or the Q fever rickettsiae. A portion of the cases of PAP is, however, set apart by the appearance of cold hemagglutinin in the serum which agglutinates homologous or type O human cells at 4° C. but not at 37° C., and an agglutinin for the MG strain of nonhemolytic streptococcus (Chap. Seventeen).

This disease was studied by Eaton and his associates, who cultured the causative agent in the chick embryo and produced pneumonia in cotton rats and hamsters by intranasal inoculation. This causative agent was considered to be a virus and was known as the Eaton agent. This microorganism appears to be an important cause of PAP, being responsible for as much as 90 per cent of the cases of the disease showing cold agglutinin. It has been found repeatedly in all parts of the country and elsewhere. and its prevalence is indicated by serological evidence of infection in undiagnosed lower respiratory infection. It was not until 1957 that the original strain was studied further, and additional, immunologically identical strains were repeatedly isolated. By means of the fluorescent antibody technique, it was shown that, following inoculation into

the amnionic cavity of 13-day embryonated eggs, the agent multiplied exclusively in the cytoplasm of the epithelial cells lining the bronchioles and air sacs of the developing embryo. It was found also that the disease may be treated effectively with tetracycline, but not penicillin. In 1962 the agent was cultivated on lifeless mediums and shown to be a Mycoplasma. The microorganism is immunologically homogeneous and has been designated M. pneumoniae. The suggestion that M. pneumoniae is an L form of the MG streptococcus⁵¹ was, perhaps, inevitable, but is not generally accepted; so far as is known the appearance of streptococcal agglutinin is fortuitous.

M. pneumoniae tends to be set apart from the other Mycoplasma in that it grows relatively slowly, forming granular colonies similar to those of M. fermentans, and in that it produces a hemolysin. It will grow in the presence of dilute (0.002 per cent) methylene blue and reduces triphenyl tetra-

zolium to give a pink discoloration,³⁶ characteristics which allow the development of selective isolation mediums.

Other infections. Mycoplasma have been associated with a number of other diseases of man, especially nongonococcal urethritis (Chap. Nineteen) in which M. hominis type 1 has been found repeatedly in large numbers. Various species have been found in conjunction with exudative pharyngitis and tonsillitis, and the former condition has been produced in human volunteers with M. hominis type 1.43 In view of the arthritis of rats and mice produced by Mycoplasma, an association with arthritis in man has been considered, but these microorganisms have been found only occasionally in arthritic synovial fluid, and the condition is not suppurative as it is in rodents. The pathogenicity of Mycoplasma, other than M. pneumoniae, for man remains uncertain as vet, although the observed associations are suggestive.

Donovania granulomatis 57

The disease granuloma inguinale (granuloma venereum) is not to be confused with lymphogranuloma inguinale of virus etiology. It is characterized by a slowly progressive ulceration in the genital region and rarely elsewhere. The initial lesion is a swelling, often in the groin as a bubo, which ruptures. Daughter lesions appear which are at first discrete and then spread slowly and coalesce, and the process may eventually involve the skin of the groin, genitals, buttocks, and lower abdomen, and the patient develops a strong fetid odor. Little effective immunity appears to be developed, at least not sufficient to appreciably arrest the progress of the infection.

Bacillary bodies, stained by Wright's stain, were observed by Donovan in 1905 in smears from lesions or in biopsy material, and have long been known as Donovan bodies. They may also be stained with 1 per cent pinacyanole in methanol, the capsules assuming a purplish pink color and the cell body a dark blue color. The Donovan body has been cultivated in the yolk sac, but not on the chorioallantois, of the developing chick embryo and in enriched mediums such as beef heart infusion²³ and is thus shown to be a cultivable microorganism to which the

name *Donovania granulomatis* has been given. 18, 19

It is a short, plump bacillus 1.5 to 4.5 μ in length and 0.8 to 1.4 μ in breadth, gramnegative, and showing prominent polar granules. Prior to cell division the elongated bacillary forms tend to become curved and may remain attached after division to give rise to chains of bacilli, the coiled filaments often seen in the usual stained preparations. The relatively heavy encapsulation observed in preparations from lesion material and the mucoid character of volk sac cultures and initial cultures on artificial mediums diminish with continued culture. Aside from its highly fastidious growth requirements, D. granulomatis closely resembles Friedländer's bacillus and has been found to be closely related immunologically to this bacterium and to other coliform bacilli. There are antigenic differences between strains, complicating the problem of serodiagnosis, as by complement fixation. 17, 20

Prior to the isolation in pure culture of *D. granulomatis*, its causal relation to granuloma inguinale was only suggested by association. Material from yolk sac cultures gives a skin reaction in persons having the disease, and a mucoid substance from in-

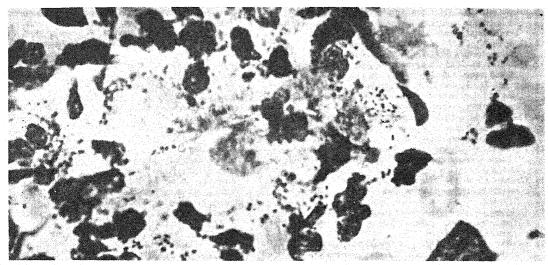


Figure 155. Donovania granulomatis in a waginal smear. The microorganisms are the small ovoid bodies both within polymorphonuclear leucocytes and lying free. Wright's stain; × 1200.

fected yolk sac, possibly a polysaccharide, gives precipitin and complement fixation reactions with serums from patients. The specificity of these reactions is, however, open to question in that apparently specific complement fixation was given by persons with chronic nonspecific ulceration perhaps attributable to the serological relation of D. granulomatis to coliform bacilli. Greenblatt and his co-workers were able to produce the disease in two human volunteers, one inoculated by a subcutaneous transplant of biopsy material and the other by the subcutaneous inoculation of yolk sac culture, but later were unable to repeat these results.¹⁰ Since it has not yet been possible to infect experimental animals other than the chick embryo, the etiological relation of D. granulomatis to granuloma inguinale cannot be regarded as fully established. Successful chemotherapy of the disease with chloramphenicol and tetracyclines has, however, been reported.

The general tendency to regard this disease as venereal is based in large part on the location of the lesions. There is little or no direct evidence that it is transmitted primarily by sexual contact and, in fact, its occurrence in both marital partners is uncommon. The probable incubation period of one to four weeks is not excessively long and should not greatly obscure histories of contact. It has been suggested that there is great individual variation in susceptibility and that natural resistance is usually of a

high order. The disease is associated with uncleanliness and is found in the Far East, Africa, and the Americas. In this country it occurs for the most part in the Negro of low economic status in the southeastern states, but it occurs elsewhere also. It is estimated that there are 5000 to 10,000 cases in the United States, and it has been found to constitute 2 to 3 per cent of venereal disease in Negro recruits. In general, however, the epidemiology of granuloma inguinale is as yet very poorly understood.

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97 - 125

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Chapter Twenty-eight

BACILLUS—THE SPORE-FORMING AEROBES

The spore-forming rod-shaped bacteria are divided into two groups on the basis of their relation to atmospheric oxygen. The genus Bacillus includes the aerobic forms, and the anaerobic types are designated Clostridium. A very large number of species of Bacillus have been described, the majority of them from soil and dust. Two species, Bacillus alvei and B. para-alvei, cause foul brood, a disease of bees. B. subtilis infects human beings only rarely, and, with this exception, B. anthracis is the only member of this large group that is pathogenic for man.

BACILLUS ANTHRACIS

Primarily a disease of lower animals transmissible to man, anthrax is of particular historical interest, for it was in his study of this disease that Koch provided the first demonstration of the causal relation between a specific bacterium and an infectious disease. The bacillus had been observed in the blood and organs of animals dving of anthrax by Davaine and Rayer in 1850 and by Pollender in 1855. In 1857 Brauell transmitted the disease by the inoculation of blood from infected animals. Conclusive demonstration of the causal relation between the bacilli and the disease, however, was the work of Koch, who in 1877 cultured the bacillus on the aqueous humor of the ox's eye, described its life history, and reproduced the disease with a pure culture of the microorganism. The importance of this discovery to the development of bacteriology has been discussed elsewhere.

Morphology and staining. The anthrax

bacillus is one of the largest of the pathogenic bacteria and ranges from 4.5 to $10~\mu$ in length and 1 to $1.25~\mu$ in breadth. The ends of the rods are often concave and somewhat swollen so that the appearance of a chain of anthrax bacilli has been compared to a jointed bamboo fishing rod. The cells occur singly and as end-to-end pairs or short chains in the body, but in culture long chains are formed. Unlike most of the sporulating aerobic bacilli they are nonmotile.

Capsules may be found on the bacilli in smears from an infected animal but are not found in culture except on mediums rich in animal protein, such as serum agar. The capsular material is not polysaccharide as it is in most bacteria, but is a high molecular weight polypeptide composed exclusively of d(-) glutamic acid (the "unnatural" 29.53 stereoisomer). This is a point of particular interest, for it is the first demonstrated natural occurrence of d(-) glutamic acid and of a polypeptide composed of a single amino acid. There is, in addition, a polysaccharide haptene present in the cell substance of the bacilli, which may be isolated from these bacilli;37 it appears to be the same in both virulent and avirulent strains, and its immunological function is not clear.

The anthrax bacillus also differs from most other aerobic pathogenic bacteria in that it forms spores which are visible as refractile bodies either free or located centrally within the cell. Their diameter does not exceed that of the vegetative cell, and hence the sporecontaining rod is not distorted. Spores are formed most abundantly at 32° to 35° C. and only under aerobic conditions, *i.e.*, not in the circulating blood of infected animals. Germination of the spore is usually polar,

PHYSIOLOGY 615

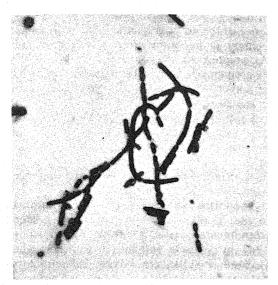


Figure 156. Bacillus anthracis, 48-hour culture on nutrient agar. The spores appear as unstained areas. Note the typical arrangement of the bacilli in coiled chains. Crystal violet stain; \times 1200.

i.e., parallel with the long axis, but rarely may be equatorial.

The bacilli stain readily, but often unevenly, with the usual aniline dyes. The granular material within the cell consists of fat, volutin, or glycogen. The bacillus is gram-positive. The spores are stained with difficulty but, after staining with hot carbol-fuchsin, are equally difficult to decolorize; hence the vegetative cells may be decolorized and stained with a contrasting dye.

The colonies of the anthrax bacillus are irregular and have a curled or hair-like structure, giving what is sometimes called a "Medusa head" appearance. On microscopic examination tangled coils of long chains of bacilli may be found. This colonial appearance is closely simulated by *B. subtilis* and some other related saprophytic, aerobic, spore-forming bacilli.

Physiology. The anthrax bacillus grows readily upon all the ordinary laboratory mediums, and growth is not improved by the addition of enriching substances. It can be grown on simple synthetic mediums; thiamin, magnesium, iron, and calcium are required together with a source of energy, and uracil, adenine, guanine, and manganese markedly stimulate growth. Growth occurs at temperatures as high as 41° to 43° C., with an optimum at 37° C. This bacillus is aerobic and facultatively anaerobic. Dextrose and trehalose are fermented rapidly but without

gas production. Sucrose, maltose, and some other carbohydrates are fermented less rapidly; lactose, galatose, mannitol, dulcitol, rhamnose, and xylose not at all. Gelatin is slowly liquefied but indol is not formed, nitrate is not reduced, and little or no hydrogen sulfide is produced. Milk is feebly acidified and is curdled by a rennet-like ferment, and the casein slowly peptonized. On potato a gray, furry growth is produced; spores are formed in particular abundance on this medium.

The anthrax bacillus may be differentiated from other species of Bacillus without difficulty except in the case of the saprophytic form, B. cereus (see differential key below), and a selective medium for the isolation of spores, which contains propagitine (0.01 per cent) and polymyxin B (20 μ g./ml.), inhibits the growth of most nonsporulating aerobic bacilli except Proteus, but does not inhibit the growth of B. cereus.25 Nevertheless, B. cereus is not an avirulent variety of the anthrax bacillus, and the two are at best only distantly related. While animal pathogenicity is the outstanding differential character of B. anthracis, it may be identified with a considerable degree of confidence by the aggregation of physiological and morphological characteristics. 10, 20, 31

The vegetative cells of the anthrax bacillus display the usual degree of resistance to deleterious influences, but the spores are relatively highly resistant, although not so resistant as the spores of B. subtilis and related forms. The bacilli have been isolated from naturally infected soil stored for as long as 60 years. 49 Anthrax spores are usually destroyed by boiling for 10 minutes and by dry heat at 140° C. for three hours. Their resistance to disinfectants is variable, for 0.1 per cent mercuric chloride may fail to kill them in 70 hours, while those disinfectants which are oxidizing agents are much more effective; 3 per cent hydrogen peroxide kills in one hour, and 4 per cent potassium permanganate in 15 minutes. In the animal carcass vegetative cells are destroyed during anaerobic putrefactive changes in 72 hours, but spores are viable under such circumstances for at least nine months. In the soil anthrax spores may remain viable for many years.

Variation. The rough variant is the virulent and naturally occurring form of *B. anthracis*. It was early noted by Pasteur that prolonged cultivation of these bacilli at

higher than optimum temperatures, 42.5° C., resulted in a loss of virulence and the appearance of asporogenous variants. A number of different types of variants are produced by such cultivation at high temperatures or in the presence of dilute antiseptics. Smooth and mucoid types of colonies have been observed, and some of these variants may be nonspore-forming. Virulence is not related to the ability to form spores, for both asporogenous virulent strains and spore-forming avirulent strains may be produced.

Virulence of the anthrax bacillus is, rather, dependent in part on the formation of the glutamyl polypeptide capsule. The bacilli of virulent R type appear rough in the conventional culture because they are not encapsulated, but they produce capsules in vivo and in vitro when the cultures are incubated under increased carbon dioxide tension in bicarbonate-containing mediums, and the colonies are mucoid in appearance. Avirulent R variants resemble the virulent form in culture in air, but the colonies remain rough and capsules are not produced on culture under increased carbon dioxide tension. 12, 45 Avirulent strains may also be encapsulated, producing mucoid colonies in air culture, but do not form toxin.

Toxins. Under ordinary conditions of culture the anthrax bacillus does not produce an exotoxin, nor is the cell substance of the microorganisms toxic. Vaccines prepared with killed bacilli do not produce an effective

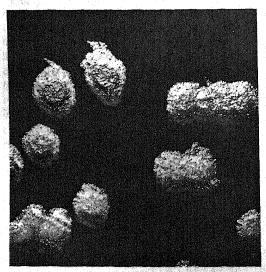


Figure 157. Colonies of *Bacillus anthracis* on nutrient agar, 24-hour culture. Note the large size and coarse texture suggestive of R variants. \times 3.

immunity, and for many years the high virulence of the anthrax bacillus and the toxemia evident in infections were not understood. As pointed out elsewhere (Chap. Nine), the discrepancy between the apparent atoxicity of a microorganism and the toxemia evident in the disease produced by it may be accounted for in two ways; *viz.*, toxicity may be produced by the microorganism only under the conditions of growth in the tissues of the infected animals, or the toxicity may be of host origin and produced by the infected tissues. ^{33, 34}

The former appears to hold true in anthrax in that a toxic substance may be isolated from infected tissue. This was first shown in 1947 by Watson and his co-workers, who isolated a substance having inflammatory activity from the tissues of infected animals. Subsequently, the biological activity present in plasma, edema fluid, and exudates in infected animals was found to be separable into two fractions by high speed centrifugation, one designated fraction I, in the sediment, and the other, fraction II, in the supernatant. Both are mildly toxic, but in combination apparently act synergistically to account for the high toxicity of the unfractionated plasma.35, 38 This toxicity is also produced in vitro in mediums containing large amounts of serum. The serum appears only to facilitate the passage of one fraction of the toxicity through sintered glass filters, and when it is not included the filtrate is atoxic. The activity adsorbed on sintered glass filters was subsequently found to be dual in nature, to give a third component of the anthrax toxin complex.4,36 At present, then, the anthrax toxin appears to contain a protective antigen (PA), an edema factor (EF), and a lethal factor (LF). None of these is toxic when tested alone, but when PA is combined with either EF or LF, the mixtures produce the edema (local) reaction or the lethal reaction respectively. These components are immunologically distinct, but their interrelationships are somewhat uncertain.24

The activity of the toxicity is not yet precisely defined,³² but it appears to be in the nature of an aggressin-like substance, having high antiphagocytic activity, and is consistent with the disease picture of anthrax and the highly invasive character of the microorganism.

Pathogenicity for lower animals.^{9, 39} In nature, anthrax is primarily a disease of

cattle and sheep; horses and swine are susceptible but are less commonly affected. In the period 1945 to 1952, 2785 outbreaks of anthrax occurred in which 14,708 domestic animals were lost in 38 states in this country. Wild deer and other gregarious herbivora are liable to occasional outbreaks.

The smaller rodents are very sensitive to inoculation.21 Rabbits, guinea pigs, and white mice are susceptible in that order. and are fatally affected by the subcutaneous introduction of a very small number of virulent bacilli. The guinea pig is often used as an experimental animal. 30 The white mouse may succumb to inoculation with a single bacillus of a highly virulent strain. Carnivorous animals, though possessing greater resistance than the herbivora, are nevertheless susceptible, as several epidemics in zoological gardens involving leopards, lions, pumas, bears, and other animals have shown. Certain animals possess a marked natural resistance to anthrax. Most rats are quite resistant,43,44 especially the white rat. The mature dog is only slightly susceptible. Birds, especially pigeons, can be infected, but not easily. Frogs are completely resistant, but toads are very susceptible.

The route by which the bacilli enter the body exerts an important influence in both experimental and natural infections. Subcutaneous inoculation is the method most commonly practiced in experimental work and is almost uniformly fatal with the ordinary small laboratory animals. Feeding experiments show that administration of spore-free cultures even to highly susceptible animals is without result, owing to the destruction of the bacilli in the stomach. The feeding of spores, on the other hand, leads to infection of the more susceptible species, although not so certainly as in subcutaneous inoculation; resistant species, such as swine, may be infected through the alimentary tract only with difficulty. Infection through the respiratory tract is possible in the experimental animal² but is probably almost unknown in the lower animals under natural conditions.

In highly susceptible animals the disease is acute and runs a rapid course; the case fatality in cattle and sheep is about 80 per cent. It presents all the characteristics of typical septicemia, and local manifestations may be almost entirely absent. Enormous multiplication of the bacteria takes place in the blood and internal organs, and sections

through the liver or spleen show the capillaries gorged with masses of bacteria. The spleen is a deep red color and greatly enlarged, hence the name splenic fever. The more resistant animal species do not develop this generalized infection, but the bacteria remain localized in an abscess or carbuncle and fail to spread through the body. This is the case with the dog and in some forms of infection in man. Such natural resistance has been found, under experimental conditions, to be separable into two components, resistance to the establishing of an infection, i.e., antibacterial resistance, and resistance to the toxin.¹⁸ Man stands perhaps midway in susceptibility between the dog and the sheep.

Under natural conditions cattle and sheep are infected through the alimentary tract by swallowing spores while grazing in infected pastures. As has been pointed out, spores are able to retain their vitality in soil for a long period, and pastures once infected may infect cattle after a lapse of as many as 30 years. In this country contaminated feed, especially bone meal, has been responsible for the infection of livestock with anthrax. Hides imported from China and other countries where the disease prevails are not uncommonly contaminated with anthrax spores; in the United States several outbreaks of anthrax among cattle with some consequent cases of human infection have been traced to the overflowing of pasture land by streams receiving the drainage of tanneries.

Cattle may also occasionally be infected by direct contact through wounds, abrasions, and other injuries to the skin; but alimentary infection is by far the most common. Anthrax has been experimentally transmitted to susceptible animals by biting flies of various species that had previously fed on animals dying from anthrax. The bacilli persist in the insects for only a short time.

Pathogenicity for man.¹⁴ Three routes of infection of human beings are known: (a) through the skin, (b) through the respiratory tract, and (c) through the alimentary tract. The bacillus is almost always transmitted to man from lower animals rather than from other human beings. The persons most commonly affected are those having to do with cattle and their products, such as butchers, shepherds, herdsmen, and handlers of hides, hair, and fleeces.^{16, 23, 50} In the United States there was a total of 400 cases of anthrax in

the period 1945 to 1955. The incidence of cases of infection from wool and hair increased nearly five-fold in the second five-year period, and over 90 per cent of the infections contracted in tanneries were from goatskins. In the period 1956–1960, 115 cases of anthrax were reported in this country.

During the First World War less efficient preliminary disinfection of hides and bristles permitted the introduction of anthrax-contaminated articles from parts of Asia and South America, and a striking increase in anthrax occurred from the use of shaving brushes-the bacilli were isolated from brushes purchased in the open market in some instances. The bacilli are destroyed on such brushes by soaking in 10 per cent formalin at 110° F, for four hours. Laboratory infections, sometimes fatal, have been known to occur with pure cultures of the anthrax bacillus. The case fatality of untreated anthrax in man is probably about 20 per cent.

Cutaneous anthrax (malignant pustule). The most common form of anthrax in the human subject is due to skin infection and usually takes the form of a localized boil or abscess, which often heals spontaneously but may progress into a septicemic condition

unless checked by incision or other surgical procedure. Owing to the relatively high resistance of man, septicemia does not often occur, especially if the carbuncle is incised and thoroughly drained. Lesions of all sizes may be produced, from a minute pustule to a large abscess.

Pulmonary anthrax. The pulmonary form of anthrax due to inhalation of the microorganisms is the most dangerous, although not the most common, variety of the disease in man. It is an occupational disease among those who handle and sort wools and fleeces and contract the infection by inhalation of spores set floating in the air from the infected material; pulmonary anthrax is known in England as "wool sorters' disease." It is characterized by many of the symptoms of pneumonia and often passes into fatal septicemia. In experimental airborne anthrax a very few spores suffice in the case of virulent strains, entering the tissues from the alveoli via the lymphatic system. The inhaled spores produce only slight local reaction except for clogging of the capillaries in the terminal stage of the disease. It is of some interest that anthrax spores may occur in the nose and throat of healthy persons exposed to inhalation infection without subsequent development of the

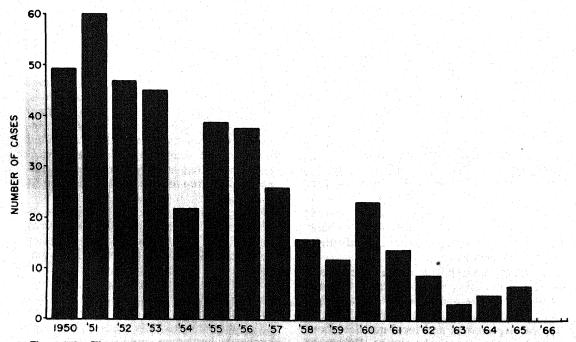


Figure 158. The number of cases of human anthrax reported in the United States during the period 1950-1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

IMMUNITY 619

disease,¹¹ and serological survey following an outbreak of pulmonary anthrax suggested the occurrence of subclinical infection.^{8, 26} There seems to be no evidence that such persons play any significant part in dissemination of the human infection, and the presence of the microorganisms probably represents no more than a transient contamination.

Intestinal anthrax. The alimentary tract, although the usual path of infection in cattle, is very rarely so in man. A few instances are on record of intestinal anthrax contracted through the medium of spore-infected food. Such cases occur among workers with animal products and have probably been due to lack of caution in handling food with uncleansed hands. Insufficiently cooked meat from anthrax-infected animals may also be a source of intestinal anthrax.

Bacteriological diagnosis. If the specimen is fresh and not grossly contaminated, the anthrax bacillus may be found in gramstained smears as a rule and is readily cultured on the usual nutrient mediums. Such a specimen may also be used for guinea pig inoculation, but it must be borne in mind that the sporulating obligate anaerobes will kill as rapidly as the anthrax bacillus. In any case the isolated culture must be tested for pathogenicity by animal inoculation. The animal will die in 36 to 48 hours after subcutaneous inoculation of a very small amount of culture. A gelatinous infiltration will be found at the site of inoculation, and the tissues of the animal contain enormous numbers of the bacilli; smears of cut spleen, for example, will show many of the large, gram-positive bacilli. This demonstration of pathogenicity is sufficient for identification, since none of the aerobic sporulating rods that resemble the anthrax bacillus are pathogenic for the guinea pig and similar experimental animals.

A precipitation test, the Ascoli test or thermoprecipitation test, is sometimes used to detect anthrax contamination of hides or other tissues. The specimen is extracted with boiling water and the extract used as an antigen in a precipitin ring test with very high titer anthrax antiserum.

Chemotherapy. Prior to the development of the sulfonamides and antibiotics, anthrax was treated with only partial success by a combination of antiserum and arsenicals. While the number of cases is limited by the

low incidence of the disease, it has been found that sulfonamides are effective chemotherapeutic agents, and penicillin remarkably so. In those instances in which the bacilli have been found to be penicillin-resistant, the tetracyclines have been found to be practically equally effective.

Immunity. 42 Natural immunity, or resistance, of certain animal species is very high. This is a consequence, at least in part, of an anthracidal activity present in tissues and associated with a histone-like protein

or polypeptide (Chap. Nine).

Recovery from infection in the susceptible animal results in a solid immunity, and the serum contains protective antibody to high titer. Nevertheless, it has not been possible to produce any appreciable immunity to challenge inoculation by inoculation with killed vaccines of anthrax bacilli, and an effective immunity has resulted only from infection with attenuated strains, as used by Pasteur in his early studies on immunity to anthrax in sheep, or by inoculation with virulent, or partially virulent, strains in conjunction with protective antiserum.

With examination of the immunological activity of the products of *in vivo* growth of anthrax bacilli, as noted above in connection with the toxin, it was found by Cromartie and his colleagues that antigen eliciting an effective immune response was produced *in vivo*, but not ordinarily in culture *in vitro*. In 1946 Gladstone found that the immunizing antigen could be produced in *in vitro* culture in the presence of body fluids containing albumin and a dialyzable fraction replaceable by sodium carbonate. The elaboration of this antigen in culture and its nature have subsequently been studied at length. ^{6, 40}

The immunizing antigen is present in cell-free filtrates of in vivo or in vitro cultures, and apparently diffuses freely from the bacterial cells. It is heat-labile and somewhat unstable but can be preserved by lyophilization, and is protein in nature. It is formed in culture in chemically defined but complex mediums containing many amino acids, purines, glutamine, thiamin, bicarbonate, and other salts.⁵² Certain of the amino acids, bicarbonate and calcium are required for formation of the antigen but not for growth.²⁷ It may be precipitated by alum,⁵¹ or salted out and purified by adsorption and elution.41 The activity is present in maximal amount in young cultures, and there is rea-

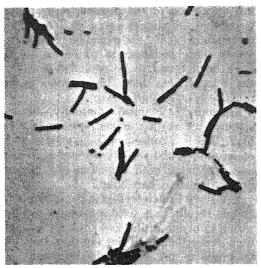


Figure 159. Bacillus subtilis, 24-hour culture on nutrient agar. No spores have formed as yet. Note the typical arrangement of the bacilli. Crystal violet stain; × 1200.

son to believe that it tends to be destroyed by bacterial polypeptidases.

This immunizing antigen is not related to the glutamyl polypeptide capsular substance, although both are required for virulence, and the antigen is produced by avirulent nonencapsulated mutants. It is intimately related to the toxin described above, but the relationship has not yet been precisely defined.

While an effective immunity is produced by prophylactic inoculation with the immunizing antigen, in man as well as monkeys and other experimental animals, the nature of the immunity so produced is not completely clear. ⁴⁷ It presumably includes antibody to the aggressin-like antiphagocytic activity and is antitoxic in that the skin lesion produced by intradermal inoculation in the rabbit is inhibited by immunization; this inhibition has been proposed as a measure of immunity to the infection.⁵

Prophylactic immunization. Active immunization of man to anthrax is not quantitatively significant under ordinary circumstances, but effective immunization of cattle, sheep, and other domestic animals can be of very considerable importance.

Immunization by inoculation with attenuated, partially avirulent bacilli, Pasteur's prophylactic, has been widely used in France for the immunization of domestic animals although infection, "vaccine anthrax," may occur. An alternative method, and one which has been used in this country in areas where

anthrax is prevalent, is Soberheim's method. It consists of the simultaneous inoculation of virulent spore suspension and immune serum from recovered animals. Either method tends to perpetuate anthrax infection.

The possibility that an effective immunity may be elicited by inoculation with sterile, cell-free preparations of the immunizing, or protective, antigen described above is of considerable practical importance. While it is fully established that an effective immunity is so produced, 3, 7, 46 in the absence of some method of quantitating the immunity the efficacy of the antigen is difficult to evaluate. The intradermal reaction noted above and correlation of protection with complement-fixing antibody titer²² have been suggested, but an immunity index has been devised¹³ which appears to be more informative and correlates with antibody titrated by gel diffusion. 19, 28 On the basis of the immunity index it has been found¹⁷ that a nonencapsulated avirulent mutant used as a vaccine gave a 10- to 15-fold increase in immunity, the protective antigen a 1000-fold increase, but immunization with protective antigen followed by inoculation with live vaccine gave as much as 109-fold increase over that of uninoculated animals. Such immunization procedures have not yet been tested on a large scale under natural conditions.

RELATED BACILLI

As indicated earlier, there are many species of aerobic sporulating bacilli closely related to and indistinguishable from the anthrax bacillus on any basis other than pathogenicity. The close morphological similarity led many of the early workers to describe "avirulent anthrax bacilli," "pseudo-anthrax bacilli," and "Bacillus anthracoides." There is, however, little basis for such differentiation. These bacilli are saprophytic soil forms and are commonly found as contaminants on plates because of the wide distribution of their spores in dust.

Of the commonly encountered forms, B. subtilis, B. megaterium, and B. cereus are among the most familiar. B. mycoides, sometimes called B. ramosus, is closely related to B. cereus and is classified as a variety of it rather than a separate species. There are some 30 species of Bacillus in all. The following portion of a key to the genus indicates the manner in which species are sepa-

rated and defined. The close relation of the anthrax bacillus to the saprophytic forms is noteworthy.

Mesophilic, aerobic bacilli with spores ellipsoidal to cylindrical and central to terminal:

- (I) Diameter of vegetative cells less than 0.9 μ (small cell variety)
 - (1) grow at pH 6.0; acetyl-methylcarbinol formed (a) gelatin hydrolyzed
 - (i) starch hydrolyzed; nitrate reduced to nitrite

Bacillus subtilis

- (ii) starch not hydrolyzed; nitrate reduced to nitriteBacillus pumilus
- (b) gelatin not hydrolyzed Bacillus coagulans
- (2) no growth at pH 6.0; acetyl-methyl-carbinol not formed
 - (a) casein digested; urease not formed Bacillus firmus
 - (b) casein not digested; urease formed Bacillus lentus
- (II). Diameter of vegetative cells 0.9 μ or more (large cell variety)
 - (1) acetyl-methylcarbinol not produced Bacillus megaterium
 - (2) acetyl-methylcarbinol produced
 - (a) saprophytic, usually motile

 Bacillus cereus

 Bacillus cereus var. mycoides
 - (b) pathogenic, nonmotile Bacillus anthracis

These forms can be distinguished from one another on the basis of details of spore formation, differential fermentations, and the like, and constitute stable types. Immunological investigation has confirmed the homogeneity of these types also. The im-

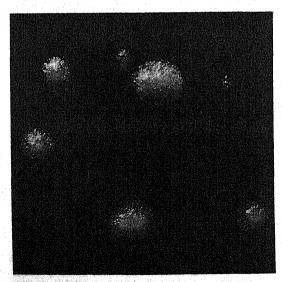


Figure 160. Colonies of *Bacillus subtilis* on nutrient agar, 24-hour culture. Note the resemblance to colonies of the anthrax bacillus. \times 3.

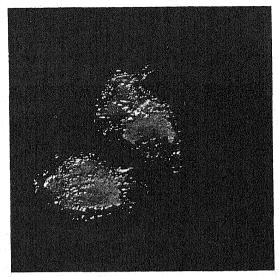


Figure 161. Colonies of *Bacillus mycoides* on nutrient agar, 24-hour culture. × 3.

munological specificity of the spores appears to be different from that of the cell substance, and four main types of the small cell group can be distinguished, though differentiation of the large cell group by this means is not so satisfactory. Similar results have been obtained in studies of the specificity of the cell substance.

The pathogenicity of these forms is very slight at best, but B. subtilis is occasionally responsible for infection,48 particularly of the eye, and rarely may produce septicemia in the immature animal. Other bacteria of this group are occasionally found to have feeble pathogenic powers. Heaslip¹⁵ isolated an aerobic sporulating bacillus, which he called B. tropicus, by the inoculation of mice with blood from persons suffering from a mild infection in Australia called "coastal fever." This bacillus has also been found there as a natural parasite of the rat and the bandicoot. It appears to be very similar to a bacillus described by Scott many years before as B. seroficus. B. alvei, the cause of foul brood of bees, is not pathogenic for man.

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Chapter Twenty-nine

CLOSTRIDIUM — THE SPORE-FORMING ANAEROBES

The group of anaerobic sporulating bacilli includes a variety of forms. 53, 77 Some of these, the anaerobic nitrogen-fixing bacteria, the butyl alcohol and acetone-producing forms, and others, have been discussed earlier (Chap. Five). Still others, however, are pathogenic for man and lower animals, and the more important of these forms—Clostridium tetani, Cl. septicum, Cl. welchii, Cl. novyi, Cl. histolyticum, Cl. chauvoei, Cl. botulinum, and the nonpathogenic but common species Cl. sporogenes—will be considered here.

The status of these bacteria as parasites is open to some question. They occur in the soil, in particular abundance in manured soils, and are found in the intestinal tract of man and animals. Cl. welchii, for example, is uniformly present in human feces, and the tetanus bacillus is often found (in up to 40 per cent of specimens examined) in the feces of domestic animals. It has been assumed by some that these bacilli are parasitic and their presence in the soil is a consequence of contamination. Although their numbers are unquestionably greatly increased by manuring and other forms of contamination, some have been found in virgin soils. It is perhaps best to regard them as essentially saprophytic soil forms that are capable of maintaining themselves in the large intestine.

None of these bacteria possesses any marked ability to invade the body tissues by itself. Cl. botulinum is apparently incapable of setting up an infection, while others, such as the tetanus bacillus, produce local infections when aided by traumatic injury to the tissues and frequently by the

presence of other bacteria. Still others, such as the bacilli associated with gaseous gangrene, show pronounced invasive properties when once established, but the initial invasion is made possible by other factors, usually trauma and the presence of other bacteria.

The pathogenicity of the anaerobic bacilli is, rather, attributable to their ability to form powerful exotoxins, a property which is curiously confined to these bacteria, the diphtheria bacillus, and the Shiga dysentery bacillus. In the case of botulism the toxin is preformed outside the animal body and, since it is unique in that it is resistant to the digestive enzymes, enters the body by way of the alimentary tract and absorption into the tissues. In the other cases a focus of infection is established, and the toxin formed at that point is disseminated through the body. In some instances, such as gangrene, an extensive local destruction of tissue occurs, but in general the diseases caused by these bacilli are essentially toxemias.

It will be clear from the foregoing considerations that infections with the sporulating anaerobes are not common under ordinary circumstances, for in most instances traumatic injury is a preliminary to infection. On the battlefield, however, such injuries are common, and tetanus and gangrene are not infrequent complications of war wounds. Such anaerobic wound infections were prominent in World War I, perhaps as a consequence of battles fought over the heavily manured fields of France. The occurrence of gaseous gangrene during World War II among troops in North Africa where the desert soil is relatively free of such forms

Morphological and	Biochemical	Characteristics	of	the
More Impor	tant Pathoge	enic Anaerobes		

		PORES CAPSULE MOTILITY			FERMENTATIONS			
	SPORES		PROTE- OLYSIS	DEXTROSE	LACTOSE	SUCROSE	EXOTOXIN	
Cl. tetani	Spherical,		+	_			3	+++‡
Cl. septicum		- <u>, -</u> ' -	+	+sl*	+	+	_	++
Cl. welchii		+		+sl	+	+	+	++
Cl. novyi		_	+	+sl	+	-	-	+++
Cl. histolyticum	Oval, sub	· · · · · · · · · · · · · · · · · · ·	+	+	a†	. —	Name of the last o	+
Cl. sporogenes	terminal	-	+	+	+	_		
Cl. chauvoei		_	+	+sl	+ .	+	+	++
Cl. botulinum			+	土	+	±	·	+++

^{*}Relatively slight.

suggests that clothing may be a more important source of contamination than had been supposed.

Cl. histolyticum is micro-aerophilic, i.e., it will grow in the presence of small amounts of oxygen, but the remainder of the forms considered here are obligate anaerobes. They may be isolated in pure culture by picking colonies from a shake culture or from plates incubated in an anaerobic jar. All are large gram-positive rods, nonencapsulated with the exception of Cl. welchii, and motile by means of peritrichous flagella with the exception of Cl. welchii. Spores are usually of a greater diameter than the vegetative cells, and the spore-containing cells are spindle- or club-shaped. The spores of the tetanus bacillus are round and termi-

nal, and those of the other bacilli oval and subterminal. Two general physiological types of anaerobic bacilli may be distinguished, the one predominantly fermentative or saccharolytic and the other predominantly proteolytic. These and other characteristics are summarized in the accompanying table.

Large amounts of volatile organic acids are produced in the fermentation of carbohydrates by these bacilli; they differ in this respect from the other pathogenic bacteria which produce nonvolatile acid, i.e., lactic acid, for the most part. Amino acids are vigorously attacked by the obligate anaerobes; some are "fermented" to organic acids, while others are mutually oxidized and reduced.

Clostridium tetani

Tetanus is a disease of man and animals characterized by spasms of the voluntary muscles. The spasms are often most marked in the muscles of the jaw and neck, hence the name "lockjaw." Tetanus bacillus was first described in 1884 by Nicolaier, who observed it in the pus taken from mice and other animals that had died after subcutaneous inoculation with small quantities of soil. Kitasato isolated the microorganism in pure culture in 1889 and established its etiologic relation to the disease. He also showed the inability of the tetanus bacillus

to invade the blood stream and showed the disease to be an intoxication. In 1890 von Behring and Kitasato laid the basis for antitoxic therapy in their discovery of diphtheria and tetanus antitoxins.

Morphology. Individual tetanus bacilli are slender, motile (20 to 30 peritrichous flagella), gram-positive, sporulating rods with rounded ends. Their common dimensions are 0.3 to 0.5μ in width and 2 to 5μ in length, but vegetative filaments of much greater length occur. The shorter forms are usually straight; filaments tend to curve in

[†]Acid.

[‡]Strong, moderate, and weak.

an undulating manner. Short chains or rods may occur. The spore is spherical and terminal and larger in diameter than the vegetative cell; spore-containing cells have a characteristic drumstick appearance. Isolated colonies in deep dextrose agar have a wooly appearance and may either be floculent or have an opaque center. Surface colonies are flat, rhizoid, or even feathery, and frequently exceed 1 mm. in diameter. Later the centers may become slightly raised. Colonies on blood agar show hemolysis.

Physiology. The tetanus bacillus develops in plain or dextrose broth and in brain, meat, agar, and gelatin mediums from which the air has been expelled by heating and excluded by some form of seal. If the depth of mediums is adequate, say 7 to 12 cm., no special seal is required, especially for the more viscous mediums. Growth occurs between 14 and 43° C.; the optimum temperature is 37° C.

The growth of *Cl. tetani* is influenced greatly by the presence of associated microorganisms. In sugar-free mediums it may be grown in mixed cultures upon the surface of culture mediums in contact with air through the absorption of oxygen by the associated aerobes. But in dextrose broth the growth of the tetanus bacillus in mixed culture is likely to be inhibited by acid

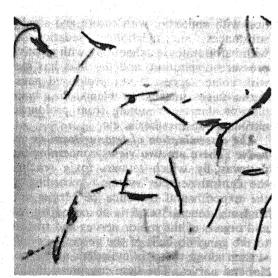


Figure 162. Clostridium tetani in pure culture. Young, actively growing culture showing beginning of spore formation. Note the refractile, unstained spores, the drumstick appearance when these are attached to the cells, and the tendency of the vegetative cells to remain attached end to end. Fuchsin; × 1150.

formation due to the associated bacteria. Sugar-free mediums, such as meat infusion peptone broth or deep meat or brain mediums, are therefore preferable for initial culture from contaminated material. Pure cultures may be isolated from these initial cultures by deep agar or surface culture methods. It has been supposed that an initial heating of the contaminated material simplifies isolation of the tetanus bacillus by destroving the vegetative cells of other microorganisms that may be present; this method may, however, leave the spores of the tetanus bacillus still badly mixed with those of other aerobic and anaerobic bacteria. some of which may be more resistant than the tetanus spores.

In pure culture, dextrose stimulates growth as in the case of the other anaerobes, although dextrose and other carbohydrates are not fermented by *Cl. tetani*. Sporulation is not inhibited by carbohydrates, as with the fermentative anaerobes; on the contrary, sporulation of the tetanus bacillus is accelerated in dextrose broth.

Growth in gelatin stab is slow at 22° C. Incubated at 37° C. for two to three days, gelatin cultures usually fail to stiffen in the refrigerator. Coagulated proteins, such as blood serum and egg white, are very slowly liquefied. Deep brain and meat mediums may be slightly softened, but never digested fully; after several weeks a slight darkening occurs near the surface exposed to air. The tetanus bacillus is, therefore, only weakly proteolytic. Amino acids are utilized readily but not by paired oxidation-reduction; glutamic acid, aspartic acid, and serine, for example, are attacked directly with the formation of CO₂, NH₃, and acetic and butyric acids. Both amino acids and carbon compounds are readily dehydrogenated. In litmus milk there is reduction of the indicator and sometimes a slight precipitation of the casein. Nitrates are not reduced to nitrites, but hydrogen sulfide and indol are produced.

The tetanus bacillus has been grown in synthetic mediums, 42 and its growth requirements are relatively complex, including the amino acids arginine, histidine, tyrosine, valine, leucine, isoleucine, and tryptophan; the bacterial vitamins riboflavin, pantothenic acid, thiamin, folic acid, biotin, pyridoxine, and nicotinic acid; the purines adenine and uracil; and oleic acid. The formation of toxin is inhibited by iron, and best yields are obtained when as much iron as possible

is taken out of the medium, though very small amounts are required.45 A beef infusion medium containing hog stomach autolysate or tryptic digest of casein, together with glucose, carbonate, potassium, magnesium, and phosphate, allows the production of toxin to high titer.

Spore formation begins in about two days at 37° C. and in eight or 10 days at room temperature. The spores are highly resistant and when protected from light and heat remain viable for years. Theobald Smith found a number of strains that resisted steaming at 100° C. for 40 to 60 minutes. Five per cent phenol is said to destroy tetanus spores in 10 to 12 hours; the addition of 0.5 per cent hydrochloric acid may reduce the time to two hours.

Antigenic structure. The tetanus bacillus is antigenically heterogeneous, and a number of types, designated by Roman numerals, have been described. At present 10 in all are known. Both somatic and flagellar antigens appear to be involved, the former of a group and the latter of a specific nature. Type VI organisms are not flagellated and lack type-specific antigen, and types II, IV, V, and IX have a common O antigen which results in higher titer cross-reactions among these than among the other types.³² Types I and III have been found most commonly in the United States, England, and France. type V in China. The toxin formed by these types is immunologically identical.

Tetanus toxin.93 As indicated elsewhere (Chap. Nine), the potent soluble toxin produced by the tetanus bacillus is of dual nature; tetanospasmin is the portion that affects the nervous tissue and tetanolysin is a hemolysin. The former is by far the more important; there is no indication that tetanolysin has any significance in connection with the ordinary symptom complex. Liquid cultures are usually toxic; 5×10^{-6} ml. may be fatal to a mouse. Although apparently diffusing freely into the surrounding medium82 the toxin is also present in the bacterial cells⁵⁵ and may be extracted from washed cells with molar sodium chloride.

The toxin is filtrable and may be freed from bacteria by filtration through Berkefeld, Chamberland, and similar filters. In aqueous solution the toxin is highly unstable to heat and light and must be stored in a cold dark place. It is apparently protein in nature, or at least has not as yet been separated from protein, and is destroyed by proteolytic enzymes and hence is ineffective when given by mouth. It may be precipitated with ammonium sulfate and in the dry state retains its potency for a long time. It is an excellent antigen and gives rise to high titer antitoxic serums.

As indicated above, tetanus toxin is one of the most potent poisons known, and its toxicity far exceeds that of alkaloids and other substances generally regarded as highly poisonous. It has been prepared in crystalline form by precipitation with methanol in the cold. These preparations contained 5 to 7.5×10^7 LD₅₀ doses for mice per mg. of nitrogen, and tetanus was produced in mice with as little as $0.000013 \text{ }\gamma$ of the crystalline toxin. Usually an incubation period intervenes between inoculation and appearance of symptoms which cannot be reduced to less than eight hours with the usual toxic filtrate, and beyond that point the incubation period is inversely related to the amount of toxin injected. With crystalline material, doses of as much as 500,000 MLD are possible in the mouse, and these large amounts produce symptoms in 30 minutes and death in one hour.

Tetanus toxin appears to act like strychnine, suppressing all types of synaptic inhibition,9 and the ultimate cause of death is asphyxia from spasm of respiratory muscles. This spasm can be released by the use of muscle relaxants and anesthetic agents. Such symptomatic treatment, in combination with antitoxin, with curare and similar substances, succinylcholine, sedation as with barbiturates, tracheotomy with positive pressure respiration, and the like, has met with some success,88 but prolonged anesthesia raises unusual problems, viz., bone marrow damage resulting from prolonged

nitrous oxide anesthesia, etc.

The dissemination of tetanus toxin in the body. There are two views concerning the pathway by which tetanus toxin reaches the central nervous system. According to the experimental evidence of Meyer and Ransom, tetanus toxin is absorbed by the end organs of the motor nerves and travels to the ganglion cells of the central nervous system along the axis cylinders of the peripheral nerves. The time consumed in this passage represents the larger part of the incubation period. The toxin may circulate for a time in the blood, but the only path to the central nervous system lies along the axis cylinders of the motor nerve tract. A

cut nerve takes up the toxin very slowly and a degenerate nerve not at all. Section of the spinal cord prevents the toxin from reaching the brain. Meyer and Ransom believed that the spinal ganglion of the sensory nerve presents a barrier to the advance of the toxin, and that for this reason sensory nerves are unable to conduct the toxin. The remarkable excitation of the motor cells of the spinal cord that is observed in tetanus is not accompanied by characteristic lesions.

Although long generally accepted, this view was challenged by Abel and his coworkers. who proposed the following theory as in accord with their experimental findings: The toxin exhibits both a central and a peripheral action, each of which may be demonstrated independently of the other. The central effect, which is characterized by reflex motor convulsions, is due to the poisoning of the motor nerve cells of the spinal cord, medulla, and pons; the peripheral effect, recognized as the unremitting rigidity of voluntary muscles, results from the fixation of the toxin upon the motor end organs. Following subcutaneous or intramuscular injection the toxin is absorbed by the lymphatics and distributed to the central nervous system by way of the arterial circulation. Normal tonicity of the motor end organs seems to be essential to the development of tetanic rigidity; neurotomy results in an immediate, sharp depression of the tonicity of the myoneural junctions, hence they are not responsive to the influence of tetanus toxin.

Abel's views have not been generally accepted in spite of their support by convincing experimental evidence which cannot be reviewed here. Other experimental data have been presented in support of the theory of the axis cylinder pathway,^{4, 91} and it may be regarded as established.

Tetanus toxin possesses a strong affinity for the cells of the central nervous system. On inoculation into the central nervous system it is rapidly fixed; the fixation time is much less than the latent period, suggesting subsequent action on the nerve tissue, particularly of the medulla. A mixture of toxin and brain substance can be injected into an animal without producing any toxic effect, the toxin apparently entering into a firm combination with some ingredient of the nerve substance. Not only the central nerve cells, but to some extent other tissue cells, are able to bind tetanus toxin. Sub-

cutaneous inoculation is less likely to be fatal than direct inoculation into nerve tissues, because some of the toxin is bound and prevented from reaching the highly sensitive nerve cells.

Pathogenicity. Tetanus is essentially an intoxication. The bacilli set up a localized infection, and the toxin formed there is disseminated through the body and gives rise to the symptom complex characteristic of the disease. Bacillemia may occur very rarely, however, and has been produced experimentally. The bacilli generally gain entrance to the tissues by means of a deep dirty wound which may be relatively small, so small sometimes as to escape serious attention. The widespread occurrence of the tetanus bacillus would seem inconsistent with the relative infrequency of tetanus infection, but mere introduction of the bacillus into the body is not sufficient to produce the disease: the microorganisms must find favorable conditions for proliferation at the site of penetration. Experimentally, pure cultures of vegetative cells or spores that have been freed from toxin cannot germinate in uninjured tissues, but simultaneous inoculation with common saprophytes or with irritant chemicals, such as calcium salts or lactic acid, enables the bacilli to grow and form toxin. As indicated elsewhere, a sufficiently low oxidation-reduction potential is necessary for the germination of tetanus spores, and it is not unlikely that the potential of normal tissues is too high to allow germination but is reduced by injury.

Tetanus in man.3, 67 Tetanus continues to be a highly fatal disease, with a case fatality rate of about 60 per cent in this country; it is claimed that symptomatic treatment as described above may reduce the case fatality rate to as little as 20 to 30 per cent. The incidence of the disease has not changed appreciably since 1947, i.e., after prophylactic immunization with toxoid had been well established by military experience, and averages about 0.3 cases and 0.2 deaths per 100,000 population. In the period 1950 to 1960 the death rate was 0.16, and an average of 465 cases were reported per year.35 Prior to 1948 the seasonal incidence reached a peak in late spring and summer, but since then has shown a peak in late summer and early fall. Half or more of the cases are reported from southern and southeastern states, with Florida having the highest rate of 1.5 and Alabama the next with

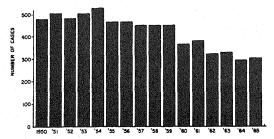


Figure 163. The incidence of tetanus in the United States as indicated by the number of cases reported during the period 1950–1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

1.3 cases per 100,000 during the period 1951 to 1954. Tetanus neonatorum (see below) is an important factor in these higher rates; in Florida during this period the non-white neonatal mortality rate was 34.3 deaths per 100,000 live births.

While as late as 1925 postoperative or surgical tetanus accounted for as much as 10 per cent of the cases, this has become relatively rare although outbreaks still occur in modern hospitals. It is most commonly associated with faulty sterilization of dressings, and occasionally results from the use of contaminated catgut. The disease may also be associated with uncommon circumstances; for example, tetanus among drug addicts, due to the use of unsterilized needles, syringes, or contaminated drugs, has been observed with some frequency in Chicago, and in a scattered way in other large cities in the United States.

By far the most common kind of tetanus is that following injury, even apparently minor or trivial injury, frequently puncture wounds which facilitate the growth of the anaerobic bacilli. Of a group of 91 cases treated in the Mayo Clinic, 85 29 were from wounds with nails and wood slivers, six other types of puncture wounds, 34 lacerations and abrasions, 17 with miscellaneous foci of infection, and in five cases there was no physical evidence of a wound. This kind of tetanus tends to occur in rural males, especially children.

Tetanus of the newborn or tetanus neonatorum is a consequence of infection of the umbilicus through septic midwifery. It is especially common among the Negroes of the southern states and in other races living under unhygienic conditions, The tonic spasms characteristic of tetanus usually begin at the site of infection, and the initial symptoms may include headache and stiffness of the neck. The spasms may remain localized in mild infections, but usually they are general and involve the whole somatic muscular system. Postmortem findings are insignificant; other than a moderate congestion, the organs show no pathological changes and the initial lesion may, of course, be inapparent or small.

The incubation period of tetanus is variable and may range from two to 50 days. The case fatality is inversely related to the incubation time; it may be as high as 70 to 80 per cent or as low as 15 to 20 per cent. Death, if it occurs, follows relatively soon after the appearance of symptoms; the dictum of Hippocrates, "such persons as are seized with tetanus die within four days, or if they pass these they recover," still stands. When the disease has a prolonged incubation period, a less sudden development of symptoms, and consequently more favorable prognosis, it is sometimes termed "chronic."

Tetanus in lower animals. Tetanus is not a rare affection in the horse, the symptoms and course of the disease being similar to the disease in man. Cattle, sheep, and hogs are less commonly affected. Experimentally, tetanus can be produced in mice and guinea pigs by the inoculation of spores introduced on splinters of wood and also by injection of toxin. The feeding of animals with tetanus bacilli, spores, or toxin is without effect. Tetanus differs from most other infectious diseases in that the diseased animal is not an appreciable factor in the spread of infection. A normal horse may distribute tetanus spores quite as widely and freely as a horse sick with tetanus.

Immunity. As indicated earlier, tetanus toxin is an excellent antigen, and high titer antitoxic serums may be prepared. Horses are good producers of antitoxin and are immunized by toxin-antitoxin mixtures followed by toxin alone. In France toxoid is used for the first injections. Because of hypersensitivities to horse serum protein, there has been some interest in human antitoxin, but the amounts available are necessarily limited.⁷³

The standardization of antitoxin. The American immunity unit is defined as "ten times the least quantity of antitetanic serum necessary to save the life of a 350 gm. guinea pig for 96 hours against the official

test dose of a standard toxin furnished by the Hygienic Laboratory of the Public Health and Marine Hospital Service." The official test dose is about 100 guinea-pig MLD's of a precipitated standard toxin. It may be noted that the L_o rather than the L+ end point is determined. Thus the tetanus antitoxic unit has slightly more than 10 times the experimental protective power of the diphtheria antitoxic unit. An antitoxic unit strength of 900 per ml. may be attained exceptionally. Practice has been different in other countries, however, and the Permanent Committee on Standardization of the Health Organization of the League of Nations found that 1 German unit, 66 American units, and 3750 French units were equivalent. It has been agreed that the international unit shall be one-half of the American unit.69

The appearance of multiple zones of precipitation has interfered with the application of the Ramon flocculation method to tetanus toxin, toxoid, and antitoxin. It has been reported that a specific flocculation zone occurs with refined enzyme-treated antitoxin. Antitoxin may also be titrated by passive hemagglutination—e.g., erythrocytes are sensitized with purified toxoid—and the correlation of such titers with those obtained by bioassay in the mouse have been reported to be very good. 84

The antitoxin deteriorates with time, the most important factor being the temperature at which it is stored. During storage for one year in the refrigerator no potency is lost, at room temperature the loss is 7 to 9 per cent, and at 37° C. it is 44 to 47 per cent.

The prophylactic use of antitoxin. 18, 79 In veterinary practice, tetanus antitoxin has been used prophylactically with a high degree of success. Vaillard collected the statistics from 1896 to 1906 of eight veterinary surgeons who inoculated 13,124 animals after operations or accidental wounds without the occurrence of a single case of tetanus. During the same time two veterinary surgeons alone saw 139 cases of tetanus among animals which did not receive the treatment. The figures of Nocard and Labat added to Vaillard's data cover the cases of 16,917 animals receiving prophylactic injections; among them only one horse had tetanus. In this case the antitoxin was given five days after the wound and the attack was mild.

It is probable that in many cases tetanus

is averted in man by the prophylactic use of antitoxin. Even though the disease is not prevented, the incubation period is delayed, and the disease may be very mild or remain localized. Precise statistical evidence involving comparable series of controls is not available, but the experience of World War I was that with increased use of tetanus antitoxin there was not only a lower incidence of the disease but an increase in mild and chronic cases. Passive immunization provides antibody in the circulating blood which combines with toxin and renders it harmless. The avidity of the nervous tissue for tetanus toxin is very great, however, and symptoms may appear in spite of the presence of circulating antibody.

Passive immunization is necessarily transient since the foreign immunoglobulin is metabolized by the recipient. The half-life of horse serum globulin in man has been calculated to be seven to 14 days, while that of human globulin, as in antitoxin of human origin, is about four weeks.⁷⁸

From time to time attempts are made to passively immunize the newborn by maternal immunization during pregnancy via transplacental antibody. In general this has not proved satisfactory, but the procedure has given encouraging results where neonatal tetanus is common.⁷⁴

The therapeutic use of antitoxin.68 Tetanus antitoxin appears to have but limited therapeutic value. It is obvious, of course, that symptoms result from damage to nerve tissue, and the administration of antitoxin will neither repair such damage nor displace the more avid nerve tissue already in combination with toxin. Reports on the therapeutic value of antitoxin are conflicting; some workers contend that intrathecal administration either alone or combined with intramuscular injection has a beneficial effect. The records of Cook County Hospital in Chicago, however, show that the therapeutic use of antitoxin has not reduced the mortality from tetanus there. It has been reported that mortality may be reduced somewhat, from 76 to 49 per cent, by the use of very large amounts of antitoxin.¹¹ Antitoxin is ordinarily given intramuscularly in man, but under experimental conditions antitoxin by the intracerebral route has therapeutic value,75 raising the question of intrathecal administration in man.

Active immunization.^{25, 71} Considerable emphasis has been placed upon active im-

munization against tetanus, particularly by the French workers. In a summary of the results of 12 years' experience with active immunization of horses and men with formol toxoid, Ramon⁷⁰ states that in one cavalry unit in which tetanus was endemic more than 50,000 horses were immunized over a 10-year period and tetanus has practically disappeared, and in a million and a half human beings immunized with toxoid no case of tetanus has occurred.

With the beginning of World War II, active immunization against tetanus was adopted by the armed services of France. Britain, and the United States. Both fluid and alum-precipitated toxoid are used, in this country the former by the Army and the latter by the Navy, and appear to be equally effective though three doses of fluid toxoid are required as against two doses of alum-precipitated toxoid. No international standard exists for tetanus toxoid; the United States Army specified that the toxin contain at least 10,000 guinea-pig MLD's per ml. and be detoxified with 0.4 per cent formalin; the final preparation must be atoxic for guinea pigs in 5 ml. amounts, and pigs receiving 1 ml. as an immunizing dose must be able to withstand 10 MLD of toxin at the end of six weeks. The importance of more than one immunizing inoculation is illustrated by the observation that the mean antitoxin titer after two inoculations was 0.35 IU (International Units), but with a third inoculation 10 months later the mean titer rose to 10 IU and 18 months later was still 0.37 IU.

More precise examination of the immunogenic potency of toxoids has shown that those prepared in different laboratories may vary widely in this respect, giving an immune response in protective units ranging from 0 to 1149 IU per ml. of serum.³⁰ An international standard has been defined²⁹ which should reduce this variability. The guinea pig is used in assaying immunogenic potency, but the use of the mouse has been urged by many.¹²

The response to booster inoculation is extremely rapid in persons giving an anamnestic reaction, so much so that booster inoculation with toxoid may be substituted

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 for prophylactic antitoxin under circumstances indicating prophylaxis. Prophylactic toxoid was given as a standard procedure to American military personnel during World War II, while British personnel received antitoxin; there was no significant difference in the incidence of tetanus between the two services.

Detectable antitoxin and ability to respond to booster inoculation may persist for as long as 15 years⁵² but it is usually recommended that booster inoculation be given at four year intervals.^{40, 47}

In practice this immunization appears to be highly effective. In the period 1942 to 1945 only 12 cases of tetanus occurred in the United States Army, of which six were in unimmunized persons; four cases occurred in the United States Navy, of which three were in unimmunized persons; in contrast a tetanus rate of about 10 per 100,000 wounded prevailed in the Japanese army and navy, which did not practice routine immunization. British experience in the Middle East war zone similarly indicated an effective immune response, though in most cases only two doses were given. The success of active immunization, coupled with the low incidence of untoward reactions (reported to be 1 in 10,000 immunizations with improved toxoid), has suggested its more general application in civil life. In France, where active immunization to tetanus has been of more and earlier interest than elsewhere, it has been made compulsory as with diphtheria immunization. Tetanus toxoid is now customarily given to children in combination with diphtheria toxoid and pertussis vaccine in this country.

Chemotherapy. The chemotherapeutic drugs are not an important adjunct in the treatment of tetanus. As pointed out above, the disease is primarily a toxemia, and the symptoms are a consequence of tissue damage by the toxin. Antitoxin is mandatory to neutralize as yet uncombined toxin, but the chemotherapeutic drugs have no antitoxic activity. There is some evidence that penicillin and tetracyclines partially protect against experimental tetanus when used prophylactically, but active or passive immunization is much more effective.

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Gaseous Gangrene 50, 60, 76

Gaseous gangrene is a syndrome which often follows dirty lacerated wounds, especially those involving fractures. It is a characteristic complication of war wounds. and present knowledge of this affection was largely developed during the First World War. But this disease is by no means so rare in civil life as was formerly thought. The increased number of injuries in automobile accidents is responsible for many cases of gangrene. Men injured about railroad tracks, either employees or vagrants, seem particularly prone to develop gaseous gangrene if not properly and promptly treated. Certain forms of peritonitis, appendicitis, intestinal obstruction, puerperal sepsis, and postoperative infections (particularly after laparotomy) are etiologically closely related to it.

The trauma usually, and perhaps necessarily, preceding the development of gangrene results in a local area of tissue anoxia, anaerobic oxidation of carbohydrate continues, and the local reducing intensity drops to levels permitting growth of the obligate anaerobes. Under the conditions of increasing acidity, the catheptic enzymes of the muscle tissue are activated, and free amino acids accumulate as a consequence of the proteolysis to provide an adequate nutrient medium for the invading microorganisms.

Gangrene is usually a mixed infection, and both aerobic and anaerobic bacteria may be isolated from a single gangrenous lesion. The aerobic and facultative anaerobic forms, such as streptococci and coliform bacilli, are not primarily concerned in the development of the pathological process but may contribute indirectly to it by facilitating the exhaustion of locally available oxygen. The gangrenous process is, rather, a consequence of the activity of the sporulating obligate anaerobes and the exotoxins produced by them. It is of more than passing interest that many of these toxins are enzymes; in addition to hyaluronidase, lecithinases, collagenases, and others are produced that have hemolytic, necrotizing, and lethal effects upon the host and host tissues.48 In the fulminating form of gaseous gangrene the muscle tissue becomes filled with gas and with a serosanguineous exudate depending for its character upon the properties of the associated microorganisms.

Of the various species of pathogenic

clostridia, Cl. welchii is usually the most frequently found, closely followed by Cl. novyi and Cl. septicum. The nonpathogen Cl. sporogenes is also found in a considerable proportion of cases; its contribution to the pathology of gangrene is uncertain and it seems probable that its common presence is a reflection of its ubiquitous distribution. Various compilations of the incidence of the several species of Clostridium found in gangrene have been made, and these do not differ significantly from one another. The frequency of occurrence of some of the more commonly found species is illustrated in the accompanying table compiled from World War II data of various authors. It is evident from this that the bacteriology of gaseous gangrene is complicated, and while there is, in effect, a typical form of gaseous gangrene, (a) it is not always produced by the same microorganism; (b) it is frequently caused by several associated agents; (c) it is often the complex result of the combined action of these principal anaerobic bacilli with various other bacteria which play an indeterminate accessory role.

THE VIBRION SEPTIQUE, CLOSTRIDIUM SEPTICUM

In 1877 and 1881 Pasteur, while studying anthrax, produced septicemia in rabbits and guinea pigs by the inoculation of putrid blood from a cow. The affection could be communicated from individual to individual, and a sporulating, motile, rod-shaped anaerobe, considered by him "one of the vibrions of putrefaction" (the actively motile bacilli sometimes appear to be curved), was regarded as the cause of the septicemia and named "vibrion septique."

In 1881 Koch described the pathological effects of a microorganism which he declared identical with the vibrion septique of Pasteur. But this bacterium failed to produce septicemia in guinea pigs, and since its pathogenic effects were limited largely to the site of inoculation, Koch designated it "the bacillus of malignant edema."

Neither Pasteur's nor Koch's description would now suffice to identify with certainty the microorganisms in question. Fortunately, the original strain of Pasteur's vibrion septique has been maintained in France, so that its outstanding characteristics are well known. The lack of any such legacy from Koch in Germany, due to his failure to recover cultures, has led to an all but interminable discussion as to the properties of the true bacillus of malignant edema. The vibrion septique is now generally known as *Cl. septicum*.

Morphology. The vibrion septique is a gram-positive, sporulating, spindle-shaped rod, or filament, and in young cultures it is motile, with many peritrichous flagella. The ends are slightly rounded and the spores, which are oval, are usually median and swell the vegetative cell into a clostridium previous to their release. Spores are formed only in mediums not containing fermentable carbohydrate in excess. The long chains and filaments of these microorganisms which occur on the visceral surfaces of infected guinea pigs are of high differential value. Capsules have never been observed. Deep colonies in 1 per cent agar are transparent or semitransparent. Hemolysis occurs on blood agar.

Physiology. Cl. septicum is a strict anaerobe and develops readily in deep brain or tissue mediums, producing gas rather abundantly. These mediums are not discolored even in the presence of metallic iron. Gelatin is liquefied, but coagulated serum and other proteins are not digested or blackened. Hydrogen sulfide is produced but indol is

Incidence of Clostridium Species in Gangrene

A Share	PER CENT OF CASES						
ORGANISM	1	2	3	4			
Cl. welchii (perfringens)	56	83	80	39			
Cl. novyi (oedematiens)	37	47	48	32			
Cl. septicum	19	24	4	_			
Cl. histolyticum	6	6	-				
Cl. tetani	13	<u> </u>	8	4			
Cl. bifermentens (sordellii)	4	35	20	54			
Cl. sporogenes	37	50	72	54			
Cl. tertium	30	59	8	3			

- 1. MacLennan: Lancet, 1943, i:63, 94, 123 (146 cases).
 - 2. Ibid., 1944, 2:203 (17 cases).
- 3. Stock: Med. Bull. E. T. O., 1944, 2:159 (25 cases).
- 4. Smith and George: J. Bact., 1946, 51:271 (110 cases).

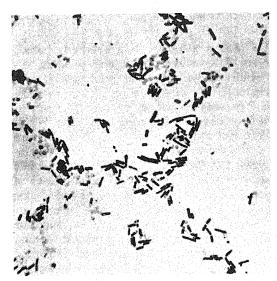


Figure 164. Clostridium septicum from pure culture. The tendency to form elongated vegetative cells is apparent. Fuchsin; \times 1050.

not. Dextrose, levulose, galactose, maltose, lactose, and salicin are fermented; mediums not containing one of these sugars support only slight growth. Sucrose, inulin, mannitol, and dulcitol are not fermented. The fermentation of salicin and nonfermentation of sucrose allow the biochemical differentiation of *Cl. septicum* and *Cl. chauvoei*, for the latter does not ferment salicin but does ferment sucrose.

Antigenic structure and toxin. Strains of Cl. septicum are immunologically related but distinct. Six groups have been distinguished on the basis of two O and five H antigens.57 These bacilli are immunologically related to Cl. chauvoei. The toxin formed, however, is specific, but a relatively weak lethal agent. The MLD for mice is about 0.005 ml. Bernheimer⁵ has prepared a dialyzable medium containing casein hydrolysate, cystine, tryptophan, glutamine, biotin, thiamin, nicotinic acid, pyridoxine, glucose, thioglycollic acid, and inorganic salts which supports the growth of some strains with the formation of 400 to 500 mouse LD₅₀ doses of toxin per ml. When injected into animals it produces a gelatinous edema and some local necrosis of the tissues. The toxin has a specific cardiac action in the cat and rabbit, producing a fall in systemic and a rise in venous blood pressure, and in the cat a specific constriction in the pulmonary and coronary circulations, with edema of the lungs and loss of fluid from the circulation. This lethal necrotic toxin, or α -toxin, is an oxygen-labile hemolysin. The toxin is neutralized by *Cl. histolyticum* antitoxin,³¹ but the affinities of the toxins are greater for homologous than for heterologous antitoxins.⁸⁰ The β -toxin is a deoxyribonuclease which attacks the nuclei of rabbit leucocytes. Hyaluronidase is formed also, and is sometimes referred to as the γ -toxin.⁵⁶

Pathogenicity. Cl. septicum does not occur in gaseous gangrene of man as frequently as some of the other anaerobic bacilli but has been found both alone and in mixed cultures. It has been recovered from gaseous infections in cattle and may be one of several microorganisms responsible for blackleg, usually considered a specific disease due to Cl. chauvoei. It has also been found in gaseous infections of hogs and other domestic animals. Experimentally the vibrion septique is strikingly pathogenic for chickens, pigeons, rabbits, guinea pigs, rats, and mice. In such animals the bacteria develop rapidly, producing gas and a reddish, serous edema. They invade the adjacent tissues and the circulation, producing septicemia, which is usually fatal within 24 to 48 hours; sublethal doses do not produce any reaction. Impression smears from the tissues, and especially from the liver, usually show elongated filaments or chains as contrasted with the single bacilli found in animals killed with Cl. chauvoei.

Antitoxic serums which are prophylactic and, to some degree, curative may be prepared by injection of *Cl. septicum* toxin into horses. The antiserums do not have the high antitoxin content that is found in antitetanic serums. Polyvalent commercial serums for prophylactic and therapeutic use in wound infections often contain septicum antitoxin.

CLOSTRIDIUM WELCHII (CLOSTRIDIUM PERFRINGENS)

Cl. welchii was first cultivated by Achalme in 1891 and supposed by him to be the cause of articular rheumatism. In 1892 Welch and Nuttall isolated this bacillus from the foamy organs of a cadaver and called it Bacillus aerogenes capsulatus. Found by Frankel the following year, it was designated B. phlegmonis emphysematosae, and in 1897 Veillon and Zuber called it B. perfringens.

Sometimes called Frankel's bacillus in Germany, Cl. perfringens in France, and Cl. welchii in English-speaking countries, it is designated Cl. perfringens by Bergey.

Morphology. Cl. welchii is a plump, nonmotile, gram-positive rod of variable length, occurring in chains and singly. Capsules are usually present in preparations made from the organs or body fluids. Spores are formed sparingly and only in the absence of fermentable carbohydrates: they are centrally located, are rarely subterminal, and do not swell the vegetative cell in which they are formed. Isolated colonies in deep agar are compact, opaque, white or grayish white biconvex discs. On blood agar the round, smooth, opaque, entire-edged colonies are relatively large, 2 to 5 mm. in diameter, and surrounded by a zone of hemolysis. An exceedingsly useful selective and differential medium containing polymyxin B and neomycin, and iron citrate to give black colonies, has been devised which allows rapid enumeration of the microorganisms.51

Physiology. Cl. welchii is a strict anaerobe and grows readily in deep brain, meat infusion broth, agar, and gelatin mediums. Growth in sugar-free mediums is restricted. Optimum conditions are provided by mediums containing fermentable carbohydrates, but such cultures are often short-lived because of the lack of spore formation and the destructive action of the formed acids on

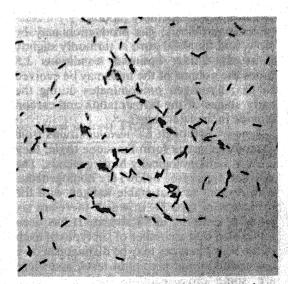


Figure 165. Clostridium welchii from pure culture. Note the relatively smaller size of these bacteria and the central spores. Fuchsin; × 1050.

the vegetative cells. Brain and meat mediums are not blackened normally, but the presence of metallic iron produces a distinct discoloration. Gelatin is liquefied, but coagulated serum or egg is not digested. Hydrogen sulfide is produced, indol is usually said to be negative, but its formation is uncertain.

Nutritive requirements are complex, and semisynthetic mediums which support growth include casein hydrolysate or 19 amino acids together with pantothenic acid, thiamin, nicotinic acid, riboflavin, biotin, folic acid, pyridoxine, adenine, guanine, uracil, inorganic salts including those of manganese and iron, together with glucose. The production of α -toxin (see below) requires two additional substances, one found in enzymatic digests of certain proteins, and the other an alcohol-soluble constituent of pancreas; glyceryl phosphocholine appears to be in part responsible for the activity of the latter.

Acid and gas are produced in glucose, maltose, lactose, and sucrose; neither mannitol nor salicin is fermented; some strains ferment inulin and some glycerol. Broth containing fermentable sugars become markedly turbid with abundant gas formation, and many cultures, possibly all at certain stages, become stringy and viscid. Milk is fermented with a characteristic "stormy" evolution of gas, followed by coagulation of the casein due to acid formation, and the curd is shortly torn to shreds by the continued evolution of gas within. The curd is not digested. This "typical" reaction is considerably modified by incomplete anaerobiosis; gas production may be slow, and the solid curd is torn only slightly if at all. Under optimum conditions 3.8 times the volume of the milk may be evolved as gas; hydrogen predominates during the early stages of the fermentation and carbon dioxide in the later stages.

Types. Although *Cl. welchii* strains from gaseous gangrene form the same toxin, they are not immunologically homogeneous. The Wilsdon types (see below) are homogeneous with respect to heat-stable antigen with the exception of type D, in which there are six kinds of antigens in 13 strains. It has been found also that a number of subtypes of each of the Wilsdon types may be distinguished by agglutination and precipitin tests, and there are some cross-reactions between types. The common antigen appears to be a capsular polysaccharide. As yet, however, sero-

logical identification of *Cl. welchii* is not practical, and gel-diffusion studies of the soluble antigens of the several types has indicated that the group tends to be heterogeneous, and there is considerable strain variation within type.²⁰ Four biochemical types have been suggested on the basis of differences in the fermentation of glycerol and inulin, but these fermentative differences do not appear to be correlated with other variable characteristics.

Toxigenic bacilli closely resembling Cl. welchii and having immunologically related toxins have been isolated from lower animals. These are the lamb dysentery bacillus (B. agni), a bacillus causing a disease of sheep called "struck" (B. paludis), and a bacillus responsible for an enterotoxemia of sheep (B. ovitoxicus). Wilsdon proposed that these be designated Cl. welchii but that four types be distinguished: Cl. welchii type A-the gaseous gangrene bacillus; Cl. welchii type B—the lamb dysentery bacillus; Cl. welchii type C-the "struck" bacillus; and Cl. welchii type D-the bacillus of enterotoxemia of sheep. Two other differentiable types were described subsequently. That designated type E has been found to be a cause of enterotoxemia of lambs and calves, and type F produces enterotoxemia of man, enteritis necroticans.19,58 Re-examination has indicated, however, that type F may not be a valid type and should be merged with type C.81 Unless otherwise indicated, however, the name Cl. welchii refers to the gaseous gangrene bacillus, or type A.

Toxins. It has been shown that, as a group, these bacilli form a number of immunologically distinct toxins and that the observed interrelationships of the toxins formed by the various types are attributable to the sharing of one or more of these components. ⁶² The effects produced by these toxins are as follows:

The α -toxin is hemolytic, lethal to mice on intravenous injection, and produces necrosis on intradermal injection into guinea pigs and rabbits. Termed by Wilsdon the W factor and designated by some workers as the ζ -toxin.

The β -toxin is not hemolytic, mice injected intravenously develop spasmodic twitchings and die almost immediately, and it produces skin necrosis in guinea pigs and rabbits. Termed by Wilsdon the Z factor.

The γ -toxin is not hemolytic, does not produce skin necrosis in guinea pigs, and is lethal to mice.

The 8-toxin is hemolytic but is not lethal to mice and does not produce skin necrosis.

The e-toxin is not hemolytic, but produces skin ne-

crosis in guinea pigs and rabbits and is lethal to mice. Termed by Wilsdon the X factor.

The θ -toxin is hemolytic and lethal and probably produces necrosis in high concentrations. It is oxygen-labile and thermolabile and very similar in properties and immunological specificity, though not identical, to streptolysin O. It is the same as Prigge's toxin.

The η -toxin has lethal activity only. It has been found in only one strain (Lechien) of type A as yet examined, and its occurrence or absence in the other types is not definitely established.

The κ -toxin is a collagenase and is necrotic on intradermal inoculation and lethal on intravenous inoculation in the rabbit. A related but differentiable activity, the λ -toxin, is not active on collagen but on altered collagen such as hide powder.

The *i*-toxin is a lethal toxin, apparently formed as a prototoxin and activated by protease activity; it is only transiently present in the growing culture.

The λ -substance is not a toxin but a proteolytic enzyme whose presence or absence is associated with type.

The μ -toxin is the name given to hyaluronidase formed by these bacilli.

The ν -substance is a deoxyribonuclease which is formed by all types of Cl, welchii.

The optimal conditions of pH, incubation time, and composition of the medium differ from one toxin to another, and a type producing more than one toxin will produce only those for which the conditions are optimal. The distribution among the Wilsdon types of the ability to form these toxins is indicated in the accompanying table. The terminology of the toxins has been somewhat confused owing to differences in the terms used by various workers; that used here has been generally agreed upon.

Of these the α -toxin has been of greatest interest, since it is associated with the virulence of the bacilli. It is a hot-cold lysin, and hemolysis is demonstrable by incubation at 37° C. for 30 minutes, followed by chilling at 2° to 4° C. The hemolysis is dependent

upon the presence of calcium or magnesium (viz., 0.0025 M calcium acetate) and is, therefore, inhibited by phosphate. The α toxin is also a lecithinase. It was observed independently by Nagler and by Seiffert in 1939 that the addition of toxic filtrate to human serum produces an opalescence, and this is known as the Nagler reaction. The opalescence is due to the splitting of lipoprotein with the liberation of free fat and has been developed as a quantitative assay of the activity by turbidity produced in saline extract of egg yolk, "lecithovitellin.36 The free lecithin is hydrolized to phosphocholine and stearyloleylglyceride; since the phosphocholine is water-soluble, the activity may be estimated by measurement of the liberated water-soluble phosphorus under standard conditions.⁴⁹ The lecithinase activity occurs at 37° C. and, unlike hemolysis, does not require subsequent chilling. The hypothesis that hemolysis is due to alteration of the erythrocyte membrane by the hydrolysis of contained lecithin is an attractive one but does not account for the necessity of subsequent chilling for hemolysis to occur. However this may be, the α -toxin has been purified by methanol precipitation in the cold and effectively separated from the other toxins of Cl. welchii; lecithinase activity, lethal activity, and hemolytic activity remained proportional, suggesting that all are activities of the same substance, but the preparations were electrophoretically heterogeneous. There is a curious inverse relationship between toxigenicity and the heat resistance and sporulating potency of the bacilli.89

The θ -toxin is sharply differentiable from the α -toxin in that it is an oxygen-labile

Toxins Formed by the Clostridium welchii Types

	TOXINS												
	TYPES		α	β	γδ	ε	θ	η ι	κ	λ	μυ		
Type A (gaseous gan	grene bacillus)	+				±	± -	+		± +		
	Bacillus agn		+	+ ; 1	+ +	+	+	? –		+.,	+ +		2015/2016 2015/2016
Type C (Bacillus pal	udis)	+	+	+ +	_	+	? –	+	,	- +		Control of the second
Type D (Bacillus ovi	toxicus)	+	-	 -	+	+	? -	. ±	±	± +		ern og engleste kennester p er okker i Corkola kolden
Type E (enterotoxem	iia)	+			_	+	? +	+	+	- +		
Type F (enteritis nec	roticans)	+	+	+			? –		الم الموسود	- +	3.44	Q., VMC.
										with the last	r Diolesia	will of	10-6-6-12-21

[±] indicates variable from strain to strain.

hemolysin, *i.e.*, is active in the reduced state, is more rapidly absorbed on sheep erythrocytes in the cold than the α -toxin, and does not require calcium or magnesium for hemolysis and is, therefore, active in the presence of phosphate.

In addition to the α -toxin and θ -toxin, the κ-toxin breaks down muscle by dissolution of its collagen and reticulin structure; the part it plays in the pathology of the infection is not clear. It does not appear to be as important as the α -toxin, for while antiserum to the α -toxin alone is protective, anticollagenase alone is not.2 All the types share with the cholera vibrio the production of myxovirus receptor-destroying enzyme. which in the cholera vibrio and the myxoviruses is neuraminidase, but evidence has been presented that in Cl. welchii it is not identical with neuraminidase.13

In addition to these toxins, there is a non-toxic antigenic substance produced, described by Fredette and his co-workers²⁴ some years ago, called the "bursting factor." It is demonstrable by enhancement of experimental infections, and produces an immunity in the absence of antitoxins.

In general the toxins formed by Cl. welchii appear to account in very large part for the observed histopathology. Robb-Smith, 72 for example, has compared the toxin in naturally occuring infection in man, in experimentally infected animals, and in normal human muscle exposed to the action of filtrates in vitro and found the histopathological changes to be substantially the same in all three.

There appears to be no simple method of typing Cl. welchii, and the presence or absence of the various toxins must be demonstrated. The typing procedure, using monospecific antiserums, is that of Oakley and Warrack referred to above, but strains do not always conform precisely to the general pattern, and cross-neutralization of the toxins may be incomplete.

Pathogenicity for man. Cl. welchii is, perhaps, the most important cause of gaseous gangrene and is found either alone or mixed with other anaerobes in the majority of cases of this disease. Tissue injury is a usual, perhaps essential, preliminary to infection, but once the bacilli are established they invade the surrounding tissue rapidly. They apparently travel along the interstitial tissue of the muscle and are often found beyond the gangrenous area. The large

amount of hyaluronidase produced would seem to be related to this rapid spread, but a number of studies have indicated that there is little or no relation between the invasiveness of *Cl. welchii* and at least *in vitro* titers of hyaluronidase.

Although most commonly found in gangrene, Cl. welchii has also been observed in closed abscesses in uterine infections and in infections of the gastrointestinal, genitourinary, and biliary tracts. It has been isolated from the blood during life, but septicemia is much less common in man than in experimental animals, although blood invasion occurs frequently in man during the agonal period or immediately following death. Study of the "foamy organs" sometimes observed at autopsy has shown that the presence of gas in the internal organs shortly after death is often attributable to an invasion by this microorganism.

Evidence has accumulated that Cl. welchii may be a common cause of food poisoning, ³⁹ similar to food poisoning of other etiology, with an incubation period of 10 to 12 hours, vomiting and diarrhea, and recovery in 24 to 48 hours (Chap. Twelve). It appears to be a foodborne infection with type A, and is unrelated to the necrotic enteritis, or pigbel, acquired from eating pork and found in Papua-New Guinea. The latter disease is a highly fatal one, with a case fatality rate of 50 per cent, apparently caused by type C (or F); serological evidence and antitoxin therapy have suggested that the β -toxin is involved in its pathogenesis. ⁵⁸

Cl. welchii is a normal inhabitant of the human intestine and is constantly present in small numbers; in fact, it has been used to a certain extent in Europe as an indicator of fecal pollution of water. The toxemia of acute intestinal obstruction has been attributed by some to the proliferation of Cl. welchii in the bowel followed by absorption of formed toxin, but it is now clear that such a relationship does not exist. This bacterium is, however, found with some frequency in gangrenous appendicitis, and it has been reported that antitoxin is of value in the treatment of perforative appendicitis.

Since infection with Cl. welchii is frequently characterized by gross blood destruction, jaundice, and anemia, a possible relationship between this bacterium and various anemias has been of some interest A pernicious and fatal anemia may be produced in experimental animals by intratibial

inoculation of culture, or a temporary but severe anemia by inoculation of filtrate. Both natural and experimental infections, therefore, lead to the development of severe anemia which is probably due to the continuous release of the hemolytic toxin.

Pathogenicity for lower animals. Natural infections in lower animals with Cl. welchii types have been referred to above. The occurrence of the gaseous gangrene bacillus, however, is rare; local abscesses have been observed in dogs and rabbits following injury. Experimentally certain strains are pathogenic for guinea pigs, pigeons, and mice, less so for rabbits. If a rabbit or guinea pig is killed a few minutes after intravenous injection of Cl. welchii and the body incubated at 37° C., gas is produced in a few hours throughout the body and the phenomenon of "foamy liver" is reproduced. This phenomenon is not strictly specific for Cl. welchii; it may be produced by similar inoculations with several other anaerobes, though the results are less striking. The pigeon is susceptible and is used for the standardization of toxin and antitoxin; an international standard has been established.21

The classic welchii toxin, the α -toxin, is not a powerful one; the MLD for a mouse is usually about 0.25 ml. of liquid culture. Antitoxins may be produced, which have both prophylactic and therapeutic value, and welchii is included in polyvalent antitoxic serums for gaseous gangrene. Antitoxin to the α -toxin appears to be far more important than that to the θ -toxin. It is difficult to develop agglutinins for Cl. welchii, and an antibacterial immunity does not protect against infection.

CLOSTRIDIUM NOVYI (CLOSTRIDIUM OEDEMATIENS)

The third important anaerobe in gaseous gangrene was probably first discovered by Novy in 1894 in a study of "malignant edema" in guinea pigs, and was designated Bacillus oedematis maligni Nr. II. It was named B. novyi by Migula in 1900. In 1915 Weinberg and Séguin isolated several strains of this bacillus, but first regarded it as a new species and named it B. oedematiens. The French name has been used by European workers.

Cl. novyi is noteworthy not only for its

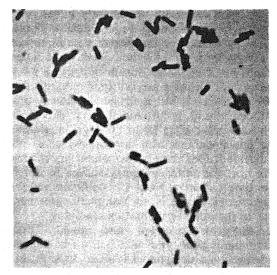


Figure 166. Clostridium novyi from pure culture. The slight tendency to curvature is apparent in some of the vegetative cells. Note the subterminal spores and the absence of large numbers of free spores. Fuchsin; × 1200.

importance in gaseous gangrene but also because of its potent exotoxin, which compares with the toxin of the diphtheria and tetanus bacilli, and for which a correspondingly potent antitoxin can be produced.

Morphology. Novy's bacillus is a large, relatively thick rod, 2.5 to 10 μ in length and 0.8 to 1 μ in breadth, and occurs singly and in chains. In cultures navicular and curved forms are found; in animal transudates the shorter form predominates. Its numerous spiral flagella, which often become tangled in "bouquets," have been emphasized in nearly all the published descriptions. The rod is nonmotile under ordinary conditions of examination, for movement is markedly inhibited in the presence of air. Subterminal spores are produced but sparsely as a rule and best in nonfermentable mediums. The bacillus is gram-positive.

Young colonies in deep dextrose agar have a yellowish, opaque, irregular center surrounded by a delicate corona of short filaments. Later the colony clears; the center becomes cloudy and in 48 hours is surrounded with a corona of tangled filaments. Surface colonies are extremely delicate, flattened, transparent, bluish gray, with irregular contours, and there is slight hemolysis on blood agar.

Physiology. Cl. novyi is a strict anaerobe and grows well at 37° C. in ordinary med-

iums and especially abundantly in the presence of a fermentable sugar. Meat and brain are not darkened; the former may be turned slightly pink or bleached. Gelatin is liquefied, but coagulated serum and egg are not digested. Nitrates are not reduced and indol is not formed, but hydrogen sulfide is produced. Acid is produced very slowly in litmus milk; after 10 to 30 days' incubation a fine flocculent clot appears which is not digested. Dextrose is fermented but lactose is not; the latter property serves to differentiate Cl. novyi from Cl. septicum and Cl. chauvoei. There is not complete agreement as to the other fermentation reactions.

Antigenic structure and toxin. Three immunological types of Cl. novyi have been defined and designated type A, type B, and type C. These correspond to types based on toxin formation (see below). Cl. hemolyticum, the etiologic agent of infectious icterohemoglobinuria of cattle, is considered to be Cl. novyi type D since it shares the β -toxin with the other types. 63

The toxin formed by this organism is the most potent of the gaseous gangrene bacilli toxins; in contrast to Cl. welchii whose filtrates contain 4 to 5 mouse MLD per ml. the lethal dose of Cl. novyi filtrate for the mouse is about 0.005 ml. Lecithinase and hemolysin activity are present, and six components have been identified by Oakley, Warrack, and Clarke.64 These are designated the α -, β -, γ -, δ -, ϵ -, and ζ -toxins, of which the α -toxin is the classic lethal toxin. The α -toxin is associated with a lipase activity which is responsible for the pearly layered colonial morphology. The lecithinase activity is biochemically similar to that of the α-toxin of Cl. welchii, but the lecithinases of types A and B are distinct serologically from one another and from the α-toxin of Cl. welchii. The distribution of the toxins corresponds to the immunological types noted above and is illustrated in the accompanying table from Oakley et al.

Pathogenicity for man. The relatively frequent occurrence of Cl. novyi in gaseous gangrene has been noted earlier. The disease is characteristically a toxemia, although septicemia is not rare. Like Cl. welchii, Novy's bacillus is often a terminal invader. In pure infections there is less tissue destruction than with Cl. welchii or Cl. septicum. The postmortem findings consist mainly in a massive localized edema, with neither

Distribution of Toxins Among Clostridium novyi Types

		CL. NOVYI TYPES					
ACTIVITY OF TOXIN	DESIG- NATION	ТҮРЕ В	TYPE C				
Lethal, necrotizing	α	+	+				
Hemolytic, necrotizing,							
lecithinase	β	_	+				
Hemolytic, lecithinase	γ	+		+?			
Oxygen-labile hemoly-							
sin	δ	+					
Opalescence in lecitho-							
vitellin	ϵ	+	_				
Hemolysin	ζ	-?	+				

the extensive gas production of the former nor the sanguineous necrosis of the latter.

Pathogenicity for animals. Natural infections due to Cl. novyi have been observed in guinea pigs, cattle, horses, and hogs. There is some evidence suggesting that type B is etiologically related to infectious necrotic hepatitis (black disease) of sheep. Guinea pigs, rabbits, rats, mice, cats, sheep, horses, and pigeons are susceptible to small doses of culture. Subcutaneous, intramuscular, and intravenous inoculations reproduce the disease experimentally. Toxicity and pathogenicity are easily lost; Novy's original strain which still survives in several laboratories, has long since failed to kill experimental animals. This is true also of strains isolated within five to 10 years.

The action of the toxin freed from bacteria is very similar to that of whole cultures. Sublethal subcutaneous doses of toxin or culture produce a peculiar nonhemorrhagic, gelatinous local edema which reaches its maximum in two or three days. It may be followed by small, superficial hemorrhages, after which it is slowly absorbed, leaving a slightly sclerotic scar. Such lesions appear not to form open phlegmons, as in the case of Cl. welchii cultures, and may also be contrasted with those of the vibrion septique and Cl. chauvoei, which, if they appear at all, are always fatal. Washed cultures are harmless.

Antitoxin has been produced in rabbits, sheep, and horses by successively increased doses of toxic filtrates. The antitoxin has prophylactic and, to some extent, therapeutic value under experimental conditions.

and is now represented in several polyvalent American serums for anaerobic infections.

CLOSTRIDIUM HISTOLYTICUM

Among the species of bacteria discovered by Weinberg and Séguin in war wounds, none is of more interest than Cl. histolyticum, so named because of its remarkable liquefying action upon living tissues. It may be somewhat more common in gaseous gangrene than indicated earlier. It has also been recovered from soil and human feces and from poisoned arrows.

Morphology. Cl. histolyticum is a grampositive motile rod, 3 to 5 μ long and 0.5 to 0.7 μ wide, that forms subterminal clostridial spores. In smears from lesions it appears generally in the form of single or paired short rods with rounded ends. The flagella, often more than 20 in number, are peritrichal. Deep agar colonies vary, according to the consistency of the medium, from compact lobulate globules in 2 per cent agar to fluffy semitransparent or even cottony balls in lower concentrations. Surface colonies are minute round dewdrops and are hemolytic on blood agar.

Physiology. Originally described as an obligate anaerobe, *Cl. histolyticum* is capable of a delicate transparent growth upon the surface of meat infusion agar and is perhaps best regarded as a micro-aerophil or as a facultative anaerobe.

This bacillus is actively proteolytic; not only is gelatin liquefied, but meat and brain and coagulated serum and egg are digested. In older cultures a precipitate of tyrosine crystals appears. Nitrates are not reduced and indol is not formed. No carbohydrates are known to be fermented in spite of statements to the contrary regarding dextrose. The action on milk is slow, but after several days a soft clot is usually formed and then slowly digested, and *Cl. histolyticum* is a proteolytic type.

Toxins. ⁵⁹ A number of toxic substances are formed, a lethal and necrotizing α -toxin and several proteolytic enzymes. One of these, the β -toxin, is a collagenase, and another, the γ -toxin is a cysteine-activated enzyme which attacks altered collagen, νiz ., azocoll. ⁶¹ A third, designated the δ -toxin, is a serologically distinct protease.

Pathogenicity. Infection with Cl. histo-

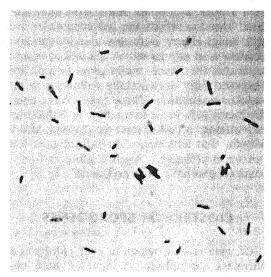


Figure 167. Clostridium histolyticum from pure culture. Note the characteristic short rods with rounded ends and the clostridial subterminal spores. Fuchsin; × 1050.

lyticum alone is probably a rare occurrence: mixed cultures with other anaerobes and aerobes appear to be the rule both in war wounds and in infections observed in horses. Most pure cultures of this bacillus are pathogenic under experimental conditions for rabbits, guinea pigs, mice, and rats, but there is considerable difference between strains. Subcutaneous inoculation of 1 or 2 ml. of a 24-hour broth culture generally produces a local tumefaction followed in 24 to 48 hours by complete sloughing of the overlying skin; then, as a rule, slow healing occurs. Intramuscular inoculation causes swelling, followed by progressive myolysis. If the gluteus muscle of a guinea pig is selected for the inoculation, it may be entirely denuded from the bone within 24 to 48 hours. The tissues literally drip away, and in some cases the limb may be disarticulated. Curiously there is often little or no evidence of intoxication of the animal, but death usually follows through peritonitis due to perforation of the peritoneum. There is occasionally invasion of the blood stream. but generally septicemia does not occur. There is no gas formation in such pure infections.

Bacteria-free filtrates have a lytic action which can be demonstrated if sufficiently large quantities (5 ml.) are injected. The most characteristic effect is the formation of a sterile hematoma filled with uncoagulated blood in which the red corpuscles are still intact. Deep intramuscular inoculation produces an edema which disorganizes and separates the tissue, while gross lesions are observed only occasionally following intravenous inoculation. Three toxins have been distinguished: an α -toxin that is lethal and necrotizing, a collagenase or β -toxin, and a γ -toxin that acts upon altered collagen but not upon collagen. Agglutinating and antitoxic serums have been produced.

CLOSTRIDIUM SPOROGENES

Cl. sporogenes, which in pure culture is a harmless saprophyte, is included here because it is frequently associated with the pathogenic anaerobes in mixed gangrenous infections, very possibly because it is so widely distributed in nature. It has frequently been confused with the pathogenic forms: not only have cultures labeled something else proved to be sporogenes, but there has been a tendency to regard "atoxic variants" or "atoxic strains" of pathogens as Cl. sporogenes. On the other hand, the presence of "atoxic variants" of pathogenic species is in many cases attributable to mixed cultures containing sporogenes. The spores of this microorganism are unusually resistant and invariably survive with those of the pathogens or even after the pathogenic spore-formers are killed in preliminary selective heating.

Morphology. Cl. sporogenes is an actively motile, gram-positive, slender rod, 3 to 7 μ in length and 0.6 to 0.8 μ in breadth, with rounded ends. The cells occur individually, in pairs, in short chains, and sometimes in filaments. The spores are oval, eccentric to subterminal, and swell the vegetative cell. The flagella are peritrichal. The deep agar colonies have the appearance of woolly balls with a dense, compact center. Surface colonies on blood agar are hemolytic, transparent, and usually rhizoid, or ameboid, with a slightly raised center; they appear moist and at first may resemble minute dew drops.

Cl. sporogenes requires strictly anaerobic conditions for growth and will grow upon all the ordinary mediums. It has been cultivated in synthetic solutions containing tryptophan, leucine, tyrosine, arginine, and

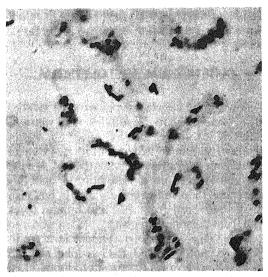


Figure 168. Clostridium sporogenes from pure culture. Note the close morphological resemblance of this species to the pathogenic forms. Fuchsin; × 1050.

phenylalanine, together with an unknown substance termed "sporogenes vitamin." Its optimum temperature is 37° C., but it will grow at temperatures as high as 50° C.

This bacillus is actively proteolytic; it produces blackening and digestion of brain and meat mediums, coagulated egg, and serum. An excess of fermentable sugar delays or inhibits this proteolysis, and the presence of metallic iron or certain iron salts accelerates it. Tyrosine crystals are not obvious. Gelatin is liquefied and blackened, hydrogen sulfide is produced, but indol production is doubtful, and nitrates are not reduced to nitrites. Reports on sugar fermentations are conflicting. According to Bergey, acid and gas are formed from dextrose, levulose, galactose, and maltose, while lactose, sucrose, salicin, and inulin are not fermented. Growth on milk is at first slow; in 48 to 72 hours a clot is formed and progressive liquefaction occurs with abundant gas and acid formation until the casein is completely peptonized.

Pathogenicity. There is no authentic record of a natural infection attributable to Cl. sporogenes alone. It has been claimed that this bacillus is a factor in certain intestinal disorders, but its frequent occurrence in the intestinal tract of healthy men and animals is not consistent with this.

In animal experiments relatively large

doses are required to produce lesions; less than 5 ml. of a young dextrose broth culture injected subcutaneously in guinea pigs, which are the most susceptible experimental animals, usually results in only a local reaction. In a few hours the immediately overlying hair loosens, the skin becomes gangrenous and raised slightly over an area of subcutaneous tissue digestion in which a small amount of gas appears. Such animals usually show no systemic involvement, and the lesion heals in a few days, leaving a necrotic scar which heals slowly. The reaction to intramuscular injection is only slightly more severe.

The most obviously important of the

pathogenic effects of Cl. sporogenes (and probably of other putrefactive anaerobes) is that of a mutual acceleration in metabolism which occurs during growth with the pathogenic anaerobes, especially Cl. welchii, Cl. septicum, and Cl. novyi. While the presence of various aerobes is in some degree stimulating to the growth of obligate anaerobes (due partly, as Pasteur suggested, to the absorption of oxygen, but also to other factors), the presence of putrefactive anaerobes greatly enhances the pathogenicity of the nonputrefactive pathogens. The proteolytic forms supply protein-split products which the fermentative types are unable to elaborate so rapidly.

Clostridium chauvoei (Clostridium feseri)

Blackleg, also known as quarter evil and symptomatic anthrax (not to be confused with anthrax), is an acute disease affecting cattle. It occurs wherever cattle are kept and is prevalent throughout the United States with the possible exception of the Southern Atlantic and Eastern Gulf States. The name blackleg, like gaseous gangrene, has been applied to affections due to various anaerobes; in some instances Cl. septicum or, rarely, Cl. novyi is found, but the principal cause is Cl. chauvoei (Cl. feseri). Just as Cl. welchii (type A) is not involved in natural infections of lower animals, so Cl. chauvoei has never been shown to be responsible for any human infection.

Although the bacilli had been earlier observed and the disease transmitted by the injection of the serous fluid from an infected animal into a healthy animal, *Cl. chauvoei* was cultivated, and its causal relation to blackleg established by Arlong, Cornevin, and Thomas in 1887.

Morphology. Cl. chauvoei is a grampositive, motile, sporulating rod. The size is variable, ranging from 3 to 8 μ in length and about 1 μ in breadth. The cells occur singly as a rule; in contrast with Cl. septicum, there is little tendency to form chains or filaments. The spores are subterminal and oval, swelling the vegetative cell in which they occur. Sporulation is often preceded by a marked swelling of the vegetative cell. Deep agar colonies are minute, compact, and downy. On the surface of blood

agar well-separated colonies are flat, round, or leaf-like, and hemolytic.

Physiology. This bacillus is a strict anaerobe and, like Cl. sporogenes, grows at temperatures as high as 50° C., though the optimum is 37° C. It will grow on the usual laboratory mediums but is best cultivated in meat or brain medium. These are neither discolored nor digested by pure cultures, but they may be slightly softened. Gelatin

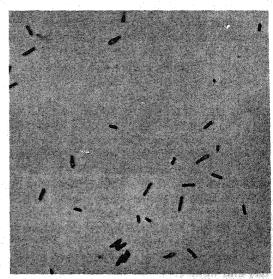


Figure 169. Clostridium chauvoei from pure culture. Subterminal oval spores are apparent; note the swollen presporulating cell on the far right. Fuchsin; × 1050.

is liquefied, but coagulated serum and egg are not. Hydrogen sulfide is produced, but indol is not formed, and nitrates are not reduced to nitrites. Dextrose, levulose, galactose, maltose, sucrose, and lactose are fermented with the formation of acid and gas; inulin, salicin, mannitol, dulcitol, and glycerol are not fermented. It may be noted again that this microorganism may be differentiated from the closely related *Cl. septicum* on the basis of the sucrose and salicin fermentation. Litmus-milk cultures become acid and the casein is precipitated, but peptonization does not occur.

Pathogenicity. Natural infections due to Cl. chauvoei occur principally in cattle. There are still several obscure features in the epidemiology. The disease occurs at special seasons of the year, is connected with certain localities, and is said to show a distinct predilection for the best young stock. The portal of entry is uncertain; whether it enters by way of minute abrasions on the skin or through the gastrointestinal mucosa is quite unknown. Experimentally, Cl. chauvoei is pathogenic for cattle, sheep, goats, guinea pigs, and mice; horses, asses,

hogs, rabbits, rats, and pigeons are somewhat refractory.

The symptoms in animals consist in crepitant localized swellings, which in natural infections occur on the thighs, neck, or shoulders. The animals become stupid, feverish, and anorectic. Treatment is rarely successful, and sick animals usually die in one to two days. The disease is a progressive bacteremia. A weak exotoxin is produced.

Immunization. An effective immunity is produced by inoculation with a formalininactivated whole culture vaccine or with washed bacteria inactivated with calcium chloride. An alum-precipitated toxoid has been used also. The immunity so produced is limited, probably effective little longer than a year, but under natural conditions of exposure to infection may be reinforced to give the appearance of a long-lasting immunity. Unlike most of the other diseases of clostridial etiology, blackleg responds to treatment with antibiotics, and penicillin is most commonly used. A hyperimmune serum may be prepared by immunization of horses with vaccine which is said to have therapeutic value.

Clostridium botulinum

Botulism was first definitely observed in Germany in 1785 and was and is associated though by no means exclusively, with the consumption of sausages; hence the not altogether appropriate name botulism. The causative bacterium was isolated by van Ermengem in 1896 and named Bacillus botulinus. It is now known as Cl. botulinum.

Morphology. Cl. botulinum is a large, pleomorphic, gram-positive, motile, sporulating rod 4 to 6 μ in length and 0.9 to 1.2 μ in breadth. The cells occur singly, in pairs, and in chains. There are four to eight peritrichal flagella. The spores are subterminal and oval and distend the vegetative cells containing them. Spore formation is variable from strain to strain, some strains producing spores abundantly, others sparsely; but in general spore formation is best in sugar-free mediums.

Deep agar colonies are translucent, globular, and diffuse, or flat and heart-shaped or disc-shaped, according to the consistency of the medium. Surface colonies are relatively large, 5 to 10 mm. in diameter, glisten-

ing, translucent at the edges with a thicker brownish center, filamentous, and hemolytic on blood agar.

Physiology. Cl. botulinum may be grown on the usual laboratory mediums under strict anaerobic conditions; cultivation on synthetic solutions has indicated that the amino acids cystine, leucine, lysine, glycine, and proline are required. Amino acids are decomposed by coupled oxidation-reduction reactions rather than by direct oxidation. Brain, meat, and coagulated protein mediums are blackened and digested; gelatin is liquefied. Milk is peptonized. Hydrogen sulfide is produced, but nitrates are not reduced to nitrites, and indol is not formed. Dextrose, levulose, and maltose are fermented; the fermentation of other sugars is variable from strain to strain and type to type. The spores are highly resistant and withstand boiling for 30 minutes to 22 hours, and autoclaving at 120° C. for as long as 20 minutes.

Irrespective of the presence of fermentable sugar, potent soluble toxins are pro-

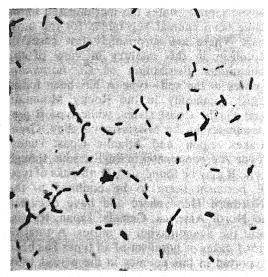


Figure 170. Clostridium botulinum type A from pure culture. Note the subterminal swollen spores and free unstained spores admixed with the vegetative cells. Fuchsin; × 1050.

duced which resemble other soluble toxins in most respects. They are, however, unusually stable to heat; heating to 80° C. for 30 minutes or boiling for 10 minutes is required for destruction. They are also relatively resistant to proteolytic digestion in the intestinal tract, and may be broken down into active fragments which may facilitate absorption from the bowel. In any case, they are unique among the classic bacterial exotoxins in that they are effective by mouth. 43, 44 They are the most potent bacterial toxins known; the guinea-pig MLD may be as small as 1×10^{-6} ml. of broth culture.

Types. Cl. botulinum is subdivided into a number of types which differ from one another in that their toxins are immunologically different. The best known of these are types A and B, which in the past have been regarded as solely responsible for human botulism. It is supposed that van Ermengem's original culture, now no longer available, was type B.

Additional types have been described since the early 1920's. A toxin-producing anaerobic bacillus isolated from fly larvae (the ingestion of which was associated with a paralytic disease of chickens) has been designated type C. A closely related bacillus was isolated from botulism of cattle in Australia which was designated B. parabotu-

linus. This bacillus is now designated Cl. botulinum type $C\beta$ and the fly larvae bacillus Cl. botulinum type $C\alpha$. The toxins of these subtypes of type C are related in that $C\alpha$ -antitoxin protects against both $C\alpha$ - and $C\beta$ -toxin, but $C\beta$ -antitoxin protects against $C\beta$ -toxin but not $C\alpha$ -toxin.

A South African strain first described by Theiler and Robinson, designated by them Cl. parabotulinum equi, was further studied and given the name Cl. botulinum type D, for its toxin is not neutralized by the antitoxins of type A, B, or C. There is some confusion in this nomenclature in that the type designated type D by the French workers corresponds to type C in this country.

Cl. botulinum type E was originally isolated from fish products which had caused human botulism, and outbreaks of human botulism associated with the consumption of fish products have occurred in this country since 1932, and in Canada and Japan. The toxin is immunologically distinct, showing no cross-neutralization by antitoxins to the other toxin types. Still another type, similarly distinct and designated type F, was described in 1958 in Denmark¹⁷ and has been isolated in this country from salmon in the Columbia River.¹⁴

Toxins. As just indicated, a number of immunologically different toxins are produced by Cl. botulinum, and a given toxin is produced by a single type rather than the various toxins being distributed among the types as in the case of Cl. welchii. Furthermore, the pharmacological action of the toxins is essentially the same and differentiation can be made only on an immunological basis, i.e., the passive protection test in which a series of experimental animals is passively immunized, each with a single antitoxin, and all challenged with the unknown toxin.

Of these toxins, that of type A is the most potent, exceeds in toxicity the other soluble toxins such as those of the diphtheria and tetanus bacilli, and is the most potent toxic substance known. All of the botulinum toxins, with the exception of type F, have been prepared in highly purified, or crystalline, form. The LD₅₀ of such preparations of type A and type B toxins contains on the order of 5×10^{-9} mg.N. As prepared, they are polymers having molecular weights of perhaps 1×10^6 , but are now known to represent aggregates of toxic moieties having molecular

lar weights of 10,000 to 12,000.^{26, 27, 28, 38} Both pure and crude botulinum toxins may be detoxified with formaldehyde to give formol toxoid which may be used for active immunization.⁹²

The types of Cl. botulinum are not biochemically or culturally distinguishable, but, as a group, they may be divided into two biochemical types, the one proteolytic (sometimes designated ovolytic but digesting proteins other than coagulated egg albumin), whose cultural characteristics have been described above, and the other saccharolytic or fermentative in character, whose members do not hydrolyze coagulated native proteins. The proteolytic group includes type A and some strains of type B (the majority of the American type B strains are proteolytic, while a great many of the European type B strains are not). The nonproteolytic group includes some strains of type B, and, so far as is known, all strains of types C, D, and E. It has been suggested that only the nonproteolytic varieties be designated Cl. botulinum and that the proteolytic varieties be termed Cl. parabotulinum.

Pathogenicity for man.⁵⁴ Human botulism is almost invariably the result of eating preserved foods in which the bacillus has grown and produced toxin. In Europe most cases have been due to the consumption of various kinds of preserved meats, such as sausage, ham, potted goose, or duck, while

in the United States the incriminated foods have been canned vegetables for the most part. There are surprisingly few cases of botulism in this country in view of the ubiquitous distribution of Cl. botulinum spores in the soil-type A has been found most commonly in the Rocky Mountain and Pacific Coast states, while type B predominates in the Mississippi Valley, Great Lakes region, and Atlantic Coast states. Type A predominates in English soils, though type B may be found also. Outbreaks of type E botulism seem to be confined to the Northern Hemisphere and have occurred in North America, Canada, Japan, Russia, and the Scandinavian countries. A total of 1561 cases of botulism of all types has been reported in this country in the period 1899 to 1963.66 The incidence is generally low; it rose to a peak of 23 outbreaks in 1922, declined, and rose again to a peak of 26 in 1935, and has since remained relatively high but with some tendency to fall.

Type E botulism has been associated with the consumption of fish and fish products. A preparation of raw fish, izushi, has been the vehicle in Japan, where 304 cases in 49 outbreaks were reported between 1951 and 1962.86 Outbreaks have occurred in Canada.16 In this country an outbreak in 1963 due to the consumption of canned tuna fish was the first instance of botulism from commercially canned foods in 40 years.41



Figure 171. The reported cases of and deaths from botulism in the United States during the period 1950-1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53, Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

Types of Clostridium botulinum

ТҮРЕ	SYNONYM	BIOCHEMICAL CHARACTER	DISEASE	ANTITOXIN
Α		Proteolytic	Botulism of man, limberneck of chickens	Specific
B		Some strains proteolytic	Botulism of man, limberneck of chickens	Specific
Сα	Fly larvae bacillus	Nonproteolytic	Paralytic disease of chickens, botulism of wild ducks	Neutralizes Cβ-toxin
Сβ	Cl. parabotulinum	Nonproteolytic	Forage poisoning of cattle (Australia)	Specific
D	Cl. parabotulinum equi	Nonproteolytic	Lamziekte of cattle (Africa)	Specific
E		Nonproteolytic	Botulism of man	Specific
F		Proteolytic	Botulism of man	Specific

Smoked fish, consumed essentially uncooked, from the Great Lakes have also produced human botulism, and the incidence of the microorganism in the intestinal contents of fish has ranged from 1 per cent in Lake Superior to 4 per cent in Lake Huron, 9 per cent in Lake Michigan, and 57 per cent in Green Bay (on Lake Michigan). Whether such contamination represents a reservoir of infection or incidental contamination from soil is not known.

As in the case of the other sporulating anaerobes, the disease produced by Cl. botulinum is an intoxication; in botulism, in fact, there is no invasion of the tissues and the toxin is preformed outside the body. Under experimental conditions in which massive doses of spores have been injected, it is probable that no infection has been set up. Cl. botulinum has, however, been found in contaminated wounds in mixed culture with aerobic and anaerobic bacteria. Under rare circumstances, then, it may proliferate in the tissues. In a group of three cases there were no symptoms of botulism, but in three fatal cases in which type A was found, symptoms of botulism were present. 15, 33, 34, 87

Human botulism is most commonly caused by types A and B. The pharmacological activity of these toxins seems to be substantially identical, and the mechanism of their action has been of considerable interest. Acetylcholine produces contraction of botulinum-poisoned muscle, but not of curare-poisoned muscle. In the poisoned animal, apparently acetylcholine is not produced in the end plates, and the action of the toxin is proximal to the point at which

it is produced. It has been found that the activity of the toxin is on the nerve filaments since acetylcholine is released following direct stimulation of the excised diaphragm of the guinea pig, but not following tetanization of the phrenic nerves. The evidence suggests that the neuromuscular paralysis observed in the poisoned animal is a result of interference with conduction in the terminal twigs of motor nerves, near or at the points of final branching, but proximal to the site of acetylcholine release.8,83 In man paralysis of the motor nerve end plates in the striated muscles and diaphragm is produced, and the symptoms include vomiting, constipation, ocular paresis, and pharyngeal paralysis.

Death may occur within a day of the onset of symptoms or may be delayed for as long as a week. At autopsy the liver, kidneys, and meninges are congested, and there may be thrombosis. The case fatality is variable; in this country it has been 60 to 70 per cent but lower in Germany, perhaps 25 per cent, possibly owing to the greater prevalence of the somewhat less toxic type B.

Pathogenicity for lower animals. Associated with human cases of botulism there have been numerous outbreaks of limberneck, a paralytic disease, among fowls fed the toxin-containing food. Other forms of botulism in lower animals occur under natural conditions. Cl. botulinum types C and D appear to be associated exclusively with the disease in lower animals. Certain forms of forage poisoning in cattle and horses in Australia are botulism, but whether the bacilli grow and form toxin in the fodder or whether the disease results from the

ingestion of rabbit carrion is not entirely clear. The South African disease of cattle, lamziekte, is botulism resulting from the ingestion of contaminated carrion. In the United States botulism of wild ducks and other waterfowl due to type $C\alpha$ is prevalent and causes the death of thousands of ducks each year. The source of the toxin ingested by these fowl is uncertain.

Experimentally rabbits, guinea pigs, mice, monkeys, cats, and dogs are susceptible to toxin administered parenterally or per os. The symptoms are similar to those of naturally infected animals and of man, and the postmortem findings are much the same. Experimental animals vary widely in their susceptibility to the toxins of the various types of Cl. botulinum.

Immunity. Formol toxoid may be used as an immunizing antigen to produce an active immunity with circulating antitoxin present in the blood. Such active immunization has been carried out in lower animals when economically feasible; in Australia botulism of sheep and cattle has assumed sufficient proportions to justify such active immunization, and it has been applied on a small scale.

Man may also be immunized with fluid or alum-precipitated toxoid of type A or type B or a mixture of both types. With an immunization schedule of 0-2-10 weeks, the arbitrarily defined protection level of 0.02 units of type A and 0.005 units of type B antitoxin per ml. of circulating blood is reached in 50 per cent of those inoculated in about three months after initiation of the immunization. Booster inoculation at one year raises the titer 500-fold the minimal protective level, and the high titer persists for at least two years.22 Under ordinary circumstances naturally occurring botulism in man is so rare that active immunization is not worth while.

Botulinum toxin is an excellent antigen, and high-titer antitoxic serums may be produced. International standards for the antitoxins have been described. Under experimental conditions these antitoxins have marked prophylactic value, but their therapeutic efficacy is slight. It may be pointed out that in botulism, as in tetanus, the symptoms are a consequence of the injury to the nerve tissue, and the administration of antitoxin serves only to neutralize circulating toxin. The almost complete leck of therapeutic effect of botulinum antitoxin in hu-

man botulism is undoubtedly attributable to the inevitable too-late administration.

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CORYNEBACTERIUM (THE DIPHTHERIA BACILLUS)

As a clinical entity diphtheria dates from the observations of Bretonneau in 1826. The diphtheria bacillus was observed and described by Klebs in 1883, but its etiological relation to the disease was suggested by the investigations of Löffler the following year. Löffler isolated the bacillus observed by Klebs in pure culture from a number of cases of diphtheria but expressly disclaimed the assumption that his bacillus was the causal agent of diphtheria, in part because he found it in the throat of a healthy child, and in part because he did not find it in all cases of what were apparently clinical diphtheria. The significance of Löffler's findings is now clear, however, for it is known that other bacteria, such as streptococci, can produce a condition in the throat closely resembling diphtheria and that the diphtheria bacillus may be present in the throat as well as in the nose in the carrier state. Further investigations by other workers indicated that the Klebs-Löffler bacillus was always present in the typical false membrane of diphtheria. In 1888 Roux and Yersin showed that this bacillus formed a soluble toxin which reproduced the characteristic symptoms and lesions of diphtheria and this demonstrated its etiological relation to the disease.

Morphology and staining.²⁰ The diphtheria bacillus is a slender rod ranging from 1 to 6 μ in length and 0.3 to 0.8 μ in breadth. The bacilli are highly pleomorphic, for, in addition to the straight or slightly curved rods, club-shaped and branching forms are not infrequently observed. Septa in the cell and the occurrence of branched forms are readily apparent in the living cell examined by phase microscopy. The presence of the latter, which are a consequence of true

branching, is indicative of the close relation of the diphtheria bacillus to some of the higher fungi. Upon completion of cell division a movement designated as snapping occurs, and the bacilli may remain attached but at sharp angles to one another.

The diphtheria bacillus tends to stain irregularly. Some cells stain solidly, others take the stain more deeply in transverse bands to give a barred appearance, and in still others deeply staining metachromatic or Babes-Ernst granules are found. A single cell may contain from one to generally not more than five or six such metachromatic granules; they may be found at one or both ends of the cells, particularly those with swollen ends, and when more than two are present the remainder are scattered within the cell substance. This irregular staining is apparent with Löffler's alkaline methylene blue or with toluidine blue.¹²

In stained smears the appearance of diphtheria bacilli is highly characteristic. They may not be identified on morphological grounds alone, for many of the pseudodiphtheria bacilli or diphtheroids also stain irregularly and are similarly pleomorphic. It was formerly thought that there was an association between morphological type and virulence. At present little emphasis is placed upon morphology in this connection, though in a general way granular types seem to predominate in clinical diphtheria, and there appears to be a rough association between morphological type and the mitis and gravis types, as will appear. The diphtheria bacillus is gram-positive but decolorizes more readily than most of the gram-positive bacteria.

Surface colonies on Löffler's serum medium or on agar are small and gray; when

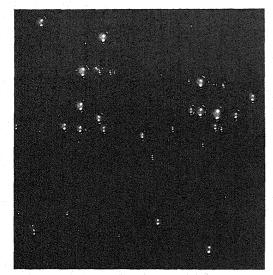


Figure 172. Colonies of Corynebacterium diphtheriae on blood agar. Note the smooth, raised, translucent appearance and relatively small size. × 2.

viewed under low magnification they are found to be coarsely granular and somewhat irregular in outline, with ragged or fringed edges. On differential mediums containing potassium tellurite, colonies of the diphtheria bacillus are dark gray or black because of reduction of the tellurite and are readily differentiated from those of contaminating bacteria. Tellurite reduction apparently occurs within the bacterial cell. Microscopic morphology may not be characteristic in smears made from tellurite medium cultures.

Physiology. The optimum growth temperature for the diphtheria bacillus is 34° to 36° C., and it grows well at 37° C.; growth will take place over the range from 15° to 40° C. An alkaline reaction is required, pH 7.8 to 8.0, and free access to air is essential, for growth under anaerobic conditions is sparse.* Growth and toxin production occur in aerated bulk, e.g., "submerged," culture in tanks.¹⁴

In primary isolation the diphtheria bacillus is best cultivated on enriched mediums. Growth is rapid on Löffler's serum medium (three parts of beef or sheep serum and one part of 1 per cent dextrose broth coagulated in slant form by inspissation), and minute but visible colonies appear after 12 to 24 hours' incubation. In recent years a variety

of differential and selective mediums have been introduced, all of which contain potassium tellurite. The better known of these are the chocolate agar-tellurite medium of Anderson and his co-workers, which has been subject to minor modifications by a number of other workers, such as Neill's medium and Hovle's medium which are used in England, and the various mediums developed by Clauberg, whose inspissated serum-glycerol-tellurite medium has been widely tested. There is general agreement that the proportion of positive cultures is somewhat higher with the Clauberg medium than with Löffler's medium; whether the heated blood-tellurite mediums are superior to Löffler's medium is not clear. As indicated above, the characteristic morphology of the diphtheria bacillus is not always seen in smears from colonies on tellurite mediums, and, therefore, in some laboratories both Löffler's medium and a differential tellurite medium are inoculated and the former used for microscopic examination if typical colonies appear on the differential medium.

The diphtheria bacillus can be cultivated on ordinary nutrient and infusion mediums. Growth is somewhat scanty on the former but good on the fresh meat infusions. Some strains, including the well-known Park 8, may be cultivated on synthetic solutions

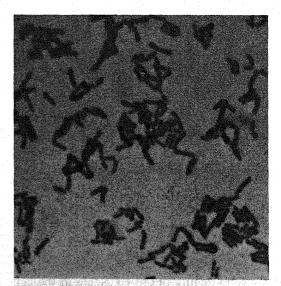


Figure 173. The diphtheria bacillus, gravis strain, pure culture on blood agar. Note the bipolar staining and the club-shaped forms. The lightly stained cells with deeply stained areas are characteristic of gravis morphology. Methylene blue stain; × 1200.

^{*}Strains of virulent diphtheria bacilli which grow more luxuriantly under anaerobic conditions than in the presence of air have been reported.

containing a number of amino acids together with small quantities of nicotinic acid, β -alanine, or pantothenic acid, and pimelic acid. It has been suggested that pimelic acid is utilized by this bacterium for the synthesis of biotin, since growth is stimulated by biotin in the absence of pimelic acid. Recently isolated strains also require oleic acid for development, especially if the inoculum is small. Nutritive requirements differ somewhat from one strain to another, and a general statement is not possible.

The diphtheria bacillus does not liquefy gelatin or digest coagulated protein. Indol is not formed,* nitrates are reduced to nitrites, and hydrogen sulfide is formed. All strains form acid but no gas from dextrose and levulose, and some strains ferment dextrin, glycogen, starch, galactose, maltose, and glycerol. There appear to be no well-defined biochemical groups among these bacilli. The fermentation of dextrose is of some interest in that propionic acid is formed. Other products of the fermentation include lactic, acetic, formic, and succinic acids and ethyl alcohol.

In ordinary culture mediums the diphtheria bacillus may remain viable for relatively long periods. It will live six to eight weeks on agar, five to six months on blood serum, 12 to 15 months on dextrose blood serum, and as long as three months in particles of diphtheritic membrane. Although virulence is ordinarily reduced by continued culture on laboratory mediums, some strains remain fully virulent, *i.e.*, toxigenic, on prolonged cultivation. The bacilli are unusually susceptible to heat; a suspension or broth culture is killed by holding at 58° C. for 10 minutes. In diphtheritic membrane they are considerably more resistant.

Antigenic structure. Although the diphtheria toxin (see below) is apparently immunologically identical in all strains, the diphtheria bacilli are heterogeneous with respect to cell antigens. They tend to agglutinate spontaneously, which may be partially countered by using 0.5 per cent saline as the suspending medium, but agglutinative types may be determined. They contain heat-stable somatic antigens which appear to be polysaccharide in nature, and heat-labile protein somatic antigens. The former have

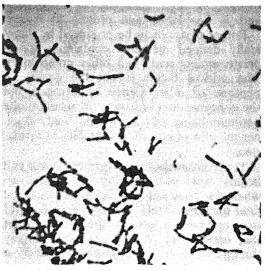


Figure 174. The diphtheria bacillus, intermedius strain, pure culture on blood agar. Note the irregular staining and barred appearance characteristic of the intermedius variety. Methylene blue stain; × 1200.

little differential value, and the agglutinative types which have been described depend upon the protein antigens.²⁴ There is some correlation between agglutinative types and the *mitis-intermedius-gravis* colonial type¹⁹ (see below), but such agglutinative types have little significance to the disease, although antibacterial immunity is considered by some to be a possible element in effective immunity (see below).

Toxin. With the exception of the Shiga dysentery bacillus, the diphtheria bacillus is the only aerobic bacterium that produces an exotoxin comparable to those formed by the sporulating anaerobes. Filtrates from broth cultures are not so toxic as those of the tetanus and botulinus bacilli; exceptionally potent filtrates may contain as much as 1000 guinea-pig MLD's per ml. Virulence is synonymous with toxigenicity, and the various virulence tests are tests for the formation of toxin.

The production of toxin is markedly influenced by environmental and nutritive conditions. A slightly alkaline reaction, pH 7.8 to 8.0, is essential; an acid reaction inhibits toxin formation. Free access to air is also necessary, and for the production of toxin the bacilli are cultivated in thin layers of beef infusion broth. Maximum amounts of toxin are found after seven to 10 days' incubation at 36° to 37° C. The toxicity is

^{*}The test with sulfuric acid and potassium nitrite may be positive because of the formation of indolacetic acid, but no color is produced with Ehrlich's reagent, p-dimethylamidobenzaldehyde.

unstable to slight acidities, pH 6.0 or less, and is heat-labile and protein in nature.

Potent toxins may be produced in chemically defined mediums containing appropriate amino acids and other compounds. The critical factor is not the quality of peptone or other source of nitrogen, but the concentration of iron in the medium; the optimum being $0.14~\mu g$. per ml., and $5.0~\mu g$. per ml. almost completely inhibits its formation

Toxin production lags behind growth and begins rapid accumulation in the medium when the bacterial population has reached near maximal levels, and concentrations of iron optimal for growth are in excess of that optimal for toxin production. Largely through the work of Pappenheimer,36 a close relationship between cytochrome b and the succinoxidase system, and toxin formation has been established. Four mols of porphyrin and one mol of toxin disappear for every four mols of iron over the optimal concentration for toxin production. It is implied, on the one hand, that the toxin may be the protein moiety of an iron-porphyrin enzyme, and on the other, experimental evidence obtained from study of the metamorphosis of cecropia silkworm larvae has suggested that the toxin may interfere in the synthesis of such respiratory enzymes. The hypothesis is attractive and plausible, but as yet lacks supporting evidence for mammalian systems.

The toxin has been prepared as a crystalline protein and subjected to intensive investigation by Pope and Stevens.³⁹ This material is not immunologically homogeneous as indicated by the presence of agglutinin in homologous antibody whose removal by absorption did not affect antitoxic activity. Heterogeneity is also indicated by application of the gel-diffusion technique, and two antigens, in addition to that of the neurotoxin, are demonstrable and are also characterized by resistance to alkaline phosphate and pepsin.

Toxicity. The diphtheria toxin is a potent poison, though not as potent as the botulinum toxins, and is primarily a neurotoxin. Highly pruified preparations have a molecular weight of about 72,000, and the lethal dose for the guinea pig is about 1 μ g. Owing to unfortunate mistakes in the preparation of diphtheria prophylactics, it is known that about 12 guinea-pig MLD's are sufficient to kill a child. The dose-response curve in

the guinea pig is very steep, so much so that the minimal lethal dose assay, adopted many years ago and persisting to the present, is practical.

The pharmacological activity of the toxin is characteristic, and the essential features of the disease are reproducible experimentally with the toxin alone. It produces degenerative changes in the heart muscle, kidneys and liver, and peripheral nerves. The pathology in the adrenals is accentuated and characteristic in the guinea pig. The immediate cause of death in the acute disease is heart failure, and damage to peripheral nerves accounts for the post-diphtheritic paralysis observed in man, and in guinea pigs receiving near-fatal doses of toxin.

Diphtheria toxin is an excellent antigen and gives rise to high titer antitoxic serums. Like the other exotoxins, it is converted to an immunogenically potent toxoid by treatment with formaldehyde.

Lysogenicity and toxigenicity.4 The observation that virulent, i.e., toxigenic, diphtheria bacilli are lysogenic (Chap. Four) and that some nontoxigenic diphtheroid bacilli could often be made toxigenic by conversion to the lysogenic state, was made by Freeman in 1951, and has been studied by a number of workers.⁵ It was found by Hewitt that temperate phages producing lysogenic toxigenic diphtheria bacilli could be of heterologous origin, i.e., staphylococci, and this significant observation has been extended by others. Toxigenicity seems not to be a physiological character transmitted phage-mediated transduction (Chap. Seven); it is possibly an expression of an altered metabolism, perhaps porphyrin metabolism, but as yet studies have been almost exclusively genetic rather than physiological. In any case, it is of interest that some 60 years after the etiology of diphtheria had apparently been fully established, it became evident that more than one microorganism is concerned.

The epidemiological implications are obvious, but are as yet unexplored. Thus the spread of the disease may be covert in some instances in the sense that it may depend in part on the dissemination of the appropriate phage, possibly carried in part by other bacteria such as staphylococci. In this connection it has been observed that there may be a geographical distribution of such phages.¹⁸

Variation. As in other groups of bacteria, smooth and rough variants of the diphtheria bacillus have been observed, and the type of colony formation has been found to be correlated with morphology and virulence; the S variant is the more virulent and the form commonly found in acute cases of diphtheria. Morphological and biochemical variation observed in the diphtheria bacilli has been critically reviewed by Morton.³²

Types²⁸ Morphological types of the diphtheria bacillus were described by Anderson and others in England in 1931 and since have been found in various parts of the world. These were of very considerable interest when first observed for there appeared to be an association, especially in England, between the type and the degree of severity in the clinical manifestations of the disease. Those designated as the gravis and intermedius types were found in severe cases of diphtheria, and the mitis type in the milder cases. The association was less clear on the Continent, and there appeared to be little or no relation in the United States, where the mitis type occurs much more frequently and perhaps only 1 per cent of the strains are of the gravis type.

The toxins produced by the three types are equally neutralizable by the ordinary antitoxic serums, but the *mitis* strains produce toxin somewhat more actively *in vitro* then the *gravis* and *intermedius* types. The three types also appear to be equally virulent for the guinea pig. By now it is more or less

generally agreed that the differentiation of these types is not significantly related to clinical severity but has been useful from an epidemiological point of view.

These types may be differentiated by their colonial form on tellurite mediums. The gravis type produces irregular striated colonies predominantly gray in color; the mitis type produces small, round, smooth, convex colonies predominantly black in color and softer in consistency; and the colonial form of the intermedius type lies between these. Colonial differences are also apparent on certain other mediums, such as trypsin-serum agar and a potato extractcystine-water blue-glycerol medium devised by Clauberg. On fresh blood agar the mitis type is usually hemoltyic, the intermedius type nonhemolytic, and the gravis type usually nonhemolytic. A further distinction is the fermentation of glycogen and starch by the gravis type, but other biochemical tests do not differentiate these types.

There is some association between colonial type and the morphology of the bacillary forms. Those of the gravis type show one or two deeply staining areas, the remainder of the cell staining very lightly; metachromatic granules are seldom observed. Bacilli of the mitis variety stain irregularly and contain very many well-developed metachromatic granules. The intermedius forms have the familar barred appearance. Whereas some 80 per cent of

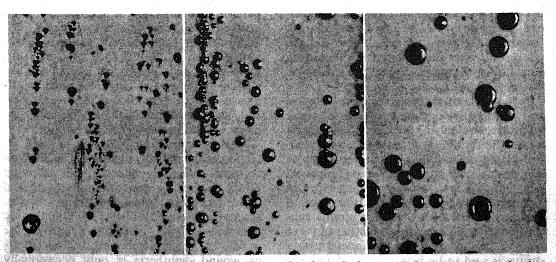


Figure 175. The varieties of the diphtheria bacillus on chocolate tellurite agar. Left, mitis type; note the characteristic raised, small black colony. Center, intermedius type; the lighter color, beginning radial striation, and small size are apparent. Right, gravis type; the gray color, larger size, raised center, and radial striation are evident.

Characteristics of the Diphtheria Bacillus Types

X	TYPE		mitis	intermedius	gravis
	Microscopic		Usually long, with many metachro- matic granules – 80 per cent typical	Usually barred, club forms common— 80 per cent typical	Short, evenly staining – 50-60 per cent typical
Morphology		Tellurite	Small, round, smooth, convex, black with grayish periphery	Small, flat, dull, gray, raised center	Large, irregular, dull, gray, raised center, radial striations
	Colonial	Chocolate Broth	Smooth, semiopaque, glistening Uniform turbidity, sometimes slightly granular, soft pel- licle	Flat, dry, opaque, slight greenish zone Finely granular turbidity	Flat, dry, matt, opaque Granular, flakes, pelliclevariable
Physiology	Fermen- tation of	Glycogen Starch			+
Immunology	Hemolys	is	+ Heterogeneous	Relatively homogeneous	± Two main types

the intermedius variety conform to this morphology, only 50 to 60 per cent of the gravis strains are typical, the remainder resembling the mitis and intermedius forms. Furthermore, 5 to 20 per cent of the mitis strains show barred forms.

Serological investigation has shown that, while these three types are antigenically distinct from one another, the types are not necessarily homogeneous. The mitis strains are heterogeneous, the gravis strains fall for the most part into two types, and the intermedius strains are relatively homogeneous, some strains showing relationship to the gravis types.

Not all strains showing the morphological and biochemical characteristics of these types are virulent, i.e., toxigenic, diphtheria bacilli. Since the diphtheria bacillus is differentiated on the basis of the formation of immunologically specific toxin, it is apparent that mitis, gravis, and intermedius types of diphtheroid bacilli occur. Furthermore, a certain proportion of toxigenic strains cannot be allocated into one or another of these types. The proportion of indeterminate strains is said to be higher when diphtheria is mild.

primarily a disease of childhood, and the age incidence is an expression of waning passive immunity of maternal origin and the development of an active immunity on the one hand, and the risk of exposure on the other. The very young child is passively protected and not exposed to great risk of infection, but by school age the immunity has disappeared in large part and risk of exposure to infection is tremendously increased with entrance into school. The adolescent and adult have acquired an active immunity as a consequence of clinical or, more commonly, inapparent infection. Thus clinical diphtheria is most common in the five to 14 age group, though the carrier state and inapparent infection are probably no more so than in the higher age groups.

Diphtheria in man is usually a local infection of the mucous surfaces. The pharvnx is most commonly affected, but infection of the larynx, or membranous croup, and nasal diphtheria, or membranous rhinitis, are not infrequently observed. Diphtheritic infections of the conjunctiva and of the middle ear are less common, and cutaneous or wound diphtheria is only occasionally observed.

Pathogenicity for man. Diphtheria is The last may assume considerable pro-

portions under certain circumstances. Ulcerative diphtheria of the skin, sometimes called desert sore or tropical ulcer, has been observed in epidemic form in Haifa, and ulcers of the deep, punched-out type occurred in troops living under combat conditions in the south and central Pacific areas during World War II.2, 21 Infection of the mucous surfaces of the genital organs is occasionally found. The invasion of other localities is rare; primary infection of the lungs and diphtheritic meningitis have been observed, and infection of the umbilicus in the newborn has been reported.

Diphtheria bacillus septicemia is occasionally observed, and the suggestion frequently occurs in the German literature that generalized infection with the diphtheria bacillus occurs more often than is generally believed.49 A few cases of acute vegetative endocarditis caused by the diphtheria bacil-

lus have been reported.37

The chief local consequence of infection is a degeneration of the epithelial cells, extending to the underlying tissues and accompanied by a profuse fibrinous exudation, and the characteristic diphtheritic membrane, containing fibrin, dead tissue cells, leucocytes, and bacteria, is formed on the affected surface. The mechanical interference of the membrane with breathing may assume significant proportions and even necessitate intubation or tracheotomy.

Although diphtheria toxin undoubtedly plays a part in the formation of the membrane, its systemic effects following absorption are by far the most important, and diphtheria is, like tetanus, essentially a toxemia. The organs most severely affected are the kidneys, heart, and nerves. A variety of lesions may be found in the kidneys, acute interstitial nephritis being the most common. The lesions in the heart consist commonly of a fatty degeneration in the muscle fibers, which may be very extensive. The diphtheritic myocarditis occurring as a complication usually, but not invariably,9,43 leaves no permanent damage. Fatty degeneration also occurs both in the myelin sheath of the peripheral nerves and in the white matter of the brain and cord. These changes in muscle and nerve account for the serious cardiac weakness often observed in diphtheria and the frequent occurrence of the more or less extensive paralysis which so commonly follows an attack of the disease. It is probable that a small amount of toxin can cause extensive damage in these tissues.

Pathogenicity for lower animals. Diphtheria is not a natural disease of lower animals. Both the local and general symptoms of human diphtheria in man can be reproduced in experimental animals. Inoculations upon the healthy mucous membrane of most adult animals lead to no changes, but if young animals are injected intratracheally, or if the mucous surface is injured before inoculation, a characteristic false membrane is produced which is histologically identical with that found in man.

The subcutaneous inoculation of a guinea pig with a sufficient amount of a young broth culture or toxic filtrate will produce death in one to four days, the time depending upon the size of the inoculum. The animal becomes obviously ill 12 to 18 hours after inoculation, and nephritic symptoms, paralytic manifestations, and other characteristics of human diphtheria are often observed. Postmortem findings include edema and possibly necrosis at the site of inoculation, congestion of the regional lymphatics and abdominal viscera, a pleural exudate, and, characteristic of diphtheritic toxemia in this animal, an enlarged and hemorrhagic condition of the adrenals. As a rule, the bacilli remain localized and are not found in large numbers in the internal organs of the infected animal. Guinea pigs that receive smaller doses and do not die by the fourth day may develop paralytic symptoms and cachexia and die later on, a condition obviously different from the acute toxemia.

Animals vary considerably in their susceptibility to infection. Rats and mice are relatively refractory; rabbits are less susceptible than guinea pigs; cats, dogs, young chicks and pigeons are highly susceptible. Paralytic manifestations appear more frequently in dogs and pigeons than in guinea pigs or rabbits and may be produced in the relatively refractory rat.1

Toxin assay. Assay of toxicity is most commonly carried out in the guinea pig17 and by intradermal inoculation in the rabbit (see below); the latter is considerably more sensitive and dependent upon a local rather than a lethal response. The baby chick is uniformly susceptible to the toxin, dying within 24 hours, and its use has been urged by a number of workers. 10, 23 The toxin is also active against various kinds of cells in culture, and tissue cultures may be used

to assay the activity.^{26, 38, 44} Any such assay may be made use of in the virulence test for toxigenicity and/or applied to the titration of antitoxin.

Bacteriological diagnosis. To establish a diagnosis of infection with diphtheria bacilli, in either case or carrier, the bacillus must be isolated and its toxigenicity demonstrated. The specimen is taken on a swab, either plain or previously dipped in sterile horse serum which is coagulated on the surface by twirling in a flame. It is best to inoculate two mediums, Löffler's serum agar and a tellurite medium such as chocolate tellurite agar; if only a single medium can be used, tellurite is preferable. A blood agar plate should be inoculated as well, both for the isolation of diphtheria-like colonies and to provide for the cultivation of hemolytic streptococci which may be present. After the plates have been inoculated a smear may be made by rolling the swab on a slide, and stained with alkaline methylene blue; it will serve to show the presence of the spirochetes and fusiform bacilli of Vincent's angina should these be present.

Diphtheria bacilli grow up in 18 to 24 hours' incubation. If the characteristic black or gray colonies appear on tellurite, smears may be made from such colonies and from the Löffler slant for microscopic examination; the morphology of the diphtheria bacillus is frequently not characteristic on tellurite, as indicated above. It is often inferred that only diphtheria bacilli grow as black colonies on tellurite medium. This is not true, for any bacterium that reduces tellurite will produce similar colonies; tellurite-reducing bacteria, other than diphtheria bacilli, from the nose and throat are usually staphylococci or micrococci, and as a rule their colonies resemble those of the mitis variety, of diphtheria bacillus but are blacker. Identification in fluorescent antibody-stained smears, of cultures or clinical material, has been described,29 but does not differentiate between toxigenic and nontoxigenic bacilli.

The virulence test. If morphologically typical bacilli are found, toxigenicity must be tested by animal inoculation. This is ordinarily carried out in the guinea pig by subcutaneous or intracutaneous inoculation. In the first instance the growth from a Löffler slant is suspended in 10 ml. saline, and 4 ml. is injected subcutaneously into each of two guinea pigs, one of which has

received 250 units of diphtheria antitoxin 24 hours previously. The diphtheria bacillus will kill the unprotected pig in three to five days, and autopsy will show local edema and the characteristic hemorrhagic enlarged adrenals, while the protected animal will survive. For the intracutaneous test the growth from a Löffler slant is suspended in 20 ml. saline, and 0.15 ml. is injected into the shaved abdominal skin of each of two pigs as above. Toxigenicity is indicated by the development of a local infiltrated lesion which shows superficial necrosis in two or three days in the unprotected pig. By the latter technique a number of tests may be carried out in the same pair of animals.

The virulence test may also be carried out in the rabbit. The growth from a Löffler slant culture is suspended in 2 to 3 ml. of sterile infusion broth, and 0.1 ml. is injected intradermally. Four hours later the animal is given 1000 units of antitoxin intravenously, and immediately afterwards a second intradermal inoculation of 0.1 ml. of the bacterial suspension is made at a site adiacent to that of the first inoculation. Reactions should be read at 72 hours. If the strain of bacteria is toxigenic, the site of the first inoculation will be a central necrotic area, usually hemorrhagic, surrounded by a zone of erythema. The inoculation of antitoxin does not affect a reaction to the first inoculation, but does specificially inhibit a reaction to the second inoculation, and the site of the latter appears as a small, pinkish papule. Eight to 10 such virulence tests may be carried out simultaneously in the same animal.

As indicated above, toxigenicity, or virulence, may also be assayed in other animals, but such virulence tests have not been generally used. An *in vitro* virulence test based on the gel-diffusion technique has been widely applied. It consists essentially in growth of the bacterial strain under consideration on an agar plate in which antitoxin has been placed in a well or trench in the agar medium. The toxin diffusing from the growth forms a line of specific precipitate with the antitoxin diffusing from the reservoir. It is necessary to include a known toxigenic strain for control purposes.

Immunity. Immunity to diphtheria resulting from recovery from a frank attack of the disease, a subclinical infection, or prophylactic inoculation, is essentially an antitoxic immunity, i.e., an immunity to the

disease rather than the infection. Although the toxin is of overriding importance, there is evidence that other toxic substances formed by diphtheria bacilli may contribute to the pathogenesis of the disease, 11 and the question of the significance of an antibacterial immunity is one of perennial interest. 8, 16, 25 The latter is complicated by the serological heterogeneity of the diphtheria bacilli noted above. Effective immunity is, however, generally considered to be primarily antitoxic in nature.

Titration of antitoxin. Assay of antitoxin is based on its specific reaction with a standardized toxin. The classic methods of assay in the guinea pig, described elsewhere (Chap. Fourteen), permit the quantitation of antitoxic activity on the basis of an arbitrarily defined unit. This is necessarily a reflection of a defined dose limit of toxin, the L₊ dose, based on death of the guinea pig, and the Lr, or reactive, dose determined by intradermal titrahon. Since the toxin consists of both toxin and toxoid. a standard antitoxin must be used to define a standard toxin reagent, and the toxin so standardized is, in turn, used to assay the unknown antitoxin in units of activity.

The proportions of toxin and toxoid in a given preparation do not affect its specific reaction with antitoxin in vitro, and either toxin or antitoxin may be titrated against a standard by specific precipitation in the flocculation test. This consists of a determination of the zone of optimal proportions, and the diphtheria toxin-horse antitoxin is the classic system in the so-called H type of flocculation reaction (Chap. Fourteen). In practice flocculation, or precipitation, occurs most rapidly and in largest amount when the toxin and antitoxin are present in optimal proportions, and assay of the flocculation, or Lf, dose is readily carried out. In the standardization of commerical antitoxin, the flocculation titration is usually carried out to facilitate the subsequent guinea pig assay required by law.

The flocculation reaction is not sufficiently sensitive to measure the very small amounts of antitoxin demonstrable by the intradermal method, and there has been some interest in the use of passive hemagglutination as a sensitive *in vitro* method. Toxin, but apparently not toxoid, is adsorbed on tannic acid-treated red cells, and these are specifically agglutinated in the presence of antitoxin^{15, 45} to give results that are

closely similar to those obtained by the intradermal method (Chap. Two) at the 30 Lr level.

The Schick test. Immunity to diphtheria, then, may be measured by the amount of circulating antitoxin present in a given individual. A skin test has been devised by Schick, and is known as the Schick test, in which a minute amount of diphtheria toxin is injected intradermally. In the nonimmune the irritant action of the toxin gives rise to local erythema followed by necrosis and desquamation, and the reaction is said to be postive. In the immune, however, the toxin is neutralized by the antitoxin that is present, the characteristic reaction does not develop, and the reaction is negative. The amount of toxin injected is usually 1/50 of a guinea-pig MLD in a volume of 0.1 or 0.2 ml.; the Permanent Standards Committee of the League of Nations specifies 1/40 MLD in 0.2 ml. and 1/50 MLD in 0.1 ml. The dilute toxin is stable in 2 per cent peptone solution, a borate buffer-gelatin solution, and glycerol-gelatin solution, though not in phenol-saline.

For many years a negative Schick test has been regarded as indicating the presence of 1/20 unit or more of antitoxin per ml. in the blood serum and a positive test less than 1/40 unit. More recent experiments, however, have indicated that the so-called Schick level of immunity is much lower than this, in the neighborhood of 1/250 to 1/500 unit of antitoxin; negative reactions have been obtained in persons with as little as 0.0005 unit.

A scarification test in which diphtheria toxin is introduced by punctate scarification rather than intradermal injection has been introduced by Reh and is called *Reh's test*. It is said to be somewhat simpler to perform than the Schick test and, when carried out with a potent toxin (with a guinea-pig MLD of 2000 per ml.), to give parallel results with the Schick test.

The question of whether the Schick test is indicative of a degree of immunity such that subsequent infection is highly improbable is one that cannot be answered a priori. Experience has shown, however, that the assumption that a Schick-negative person is, for all practical purposes, immune, is pragmatically sound.

Prophylactic immunization.^{22,46} It was early observed that experimental animals can be immunized to diphtheria by the in-

jection of living cultures of the bacilli after a protective dose of antitoxic serum or by the inoculation of toxin neutralized with antitoxin.

Toxin-antitoxin. The mixture usually used contains 0.1 L₊ dose of toxin per ml. The toxin is slightly underneutralized (5 ml. of the mixture should produce diphtheritic paralysis in 300 gm. guinea pigs) but depends for its immunizing efficiency not on the slight excess of toxin but on a slow dissociation of the toxin-antitoxin complex to liberate free toxin. Administered in three doses of 1 ml. each at intervals of one to two weeks, toxin-antitoxin produces an immunity in 85 per cent of individuals inoculated. The immunity develops slowly, and one to six months may be required for the Schick reaction to become negative. Accidents may occur as a consequence of dissociation of the toxin-antitoxin mixture freezing in one instance produced such dissociation - but these are rare, particularly with the 0.1 L₊ dose mixture. There is the possibility of sensitization of the inoculated individual to horse serum.

Toxoid. The use of formol toxoid or anatoxin as an immunizing agent was introduced by Ramon in 1923 and has been widely adopted. As pointed out elsewhere (Chap. Nine), toxin treated with formaldehyde (in this case a potent toxin of more than 15 Lf doses per ml. is incubated with 0.3 to 0.4 per cent formalin at 37° C. for one month) loses its toxicity but retains its antigenicity and is a highly efficient immunizing agent. The administration of this material in three doses of 0.5, 1.0, and 1.0 ml. at intervals of two to three weeks renders 95 per cent of persons Schick-negative. It was at first thought that toxoid might entirely replace toxin-antitoxin as an immumizing agent, but this has not proved to be the case. Reactions to the bacillary protein, while not of great importance as a rule in young children, may be relatively severe in older persons, and its use is preferably restricted to children under 12 years of age. Reactivity may be tested for by the intradermal injection of toxoid—the Moloney

Toxoid-antitoxin floccules (the precipitate coming down at the optimal antigen-antibody ratio) have been used in England to a considerable extent. There is, presumably, a partial purification of the toxoid by precipitation with antibody. Another kind of

preparation that has been used in England is toxoid absorbed on aluminum phosphate, diphtheria phosphate toxoid or DPT. This material and toxin-antitoxin floccules have not been widely used in the United States. Toxoid precipitated with protamine appears to be an effective immunizing agent without giving the untoward reactions sometimes observed with alum-precipitated toxoid.

Alum-precipitated toxoid. It has been found that toxoid precipitated with potassium alum (small amounts, 1 to 2 per cent, are required) is superior as an immunizing agent to ordinary formol toxoid. Present preparations are treated with charcoal prior to alum precipitation to remove color and extraneous nitrogenous material. The precipitate is insoluble (it may be redissolved in sodium citrate or sodium tartrate) and remains in the subcutaneous tissue for a considerable time, to provide a prolonged antigenic stimulus. In spite of this, a single inoculation is not sufficient—as little as 11 per cent Schick conversion has been observed, together with a tendency to reversion—but two inoculations are as effective as three of fluid toxoid. Alum toxoid has the same tendencies to produce untoward reactions in older persons that are observed with formol toxoid.

Passive immunity. Susceptible, i.e., Schick-positive, individuals may be passively immunized to diphtheria by the injection of antitoxic horse serum or purified preparations of antitoxin. Such immunity is of relatively short duration and is not effective for longer than two or three weeks at the most.

The therapeutic use of antitoxin. Serum therapy is more successful in diphtheria than in any other disease, and there is no question of its efficacy in reducing case fatality rates. As in the case of tetanus and botulism, the therapeutic administration of antitoxin cannot bring about repair of tissues already damaged by toxin. Early administration is, therefore, essential, and there is progressive increase in the case fatality rate with each day's delay. There is no limit, beyond the volume, to the number of units that may be safely injected. Antitoxin is generally administered intramuscularly but in severe cases may be given intravenously. It is completely ineffective when given by mouth.

Usually horse serum contains 500 to 700 units per ml. and exceptionally 1000 to 1500. Concentration of the antitoxin by salting out and other procedures is generally practiced,

for, although some antitoxin is lost in the process, the concentration is increased with a corresponding reduction in the volume to be injected.

Epidemiology.⁴² The epidemiology diphtheria is considerably better understood than that of any other disease, in part because the causative agent can be isolated with relative facility from infected individuals, and in part because the Schick test allows the differentiation of the immunes and the nonimmunes. As in the case of other respiratory diseases, infectious material leaves the body in the secretions of the nose and throat, is transmitted from man to man by contact or infective droplets, and enters the body via the mouth and nose. Furthermore, the diphtheria bacillus is disseminated not only by persons with the disease but also through the agency of healthy carriers in whom there is no clinical evidence of infection. Unlike many of the diseases of the respiratory tract diphtheria is an immunizing disease, and prolonged or repeated contact with the bacillus results in the development of a solid immunity to the disease in its clinical manifestations.

Immunity and susceptibility. Schick testing indicates that while susceptibility is low in the first six months of life, owing to passive immunization with maternal antibody, the proportion of Schick-positives increases rapidly and is at a maximum in children under four or five years of age, then gradually declines in an unimmunized population. When immunization of children is widely practiced the prevalence of effective levels of antitoxin declines at about age 15 in some population groups, but not in others, presumably as a consequence of a greater carrier rate in the latter.¹³

Carriers. As indicated above, healthy individuals may harbor virulent diphtheria bacilli in their throats. These carriers need be neither immunes nor convalescents and are, for the most part, casual carriers. There is no precise information concerning the duration of this transient carrier state; it may possibly be about two weeks.

The proportion of carriers has been investigated by a number of workers. In a study of Baltimore schoolchildren, Doull and Fales found an average carrier rate of 2.32 per cent from November to May. On the basis of this Frost has estimated the carrier incidence in the five to 14 age group in that city to be 2538 per 10,000. At this

rate 75 per cent of the population becomes infected at least once in five years, 95 per cent in 10 years, and over 99 per cent in 15 years, while very considerable proportions would suffer repeated infections, the average being 2.5 infections per person in 10 years. Others have recorded considerably higher carrier rates; Dudley has reported 6.6 per cent in a boys' school, and repeated swabbings showed that at least 40 per cent carried the diphtheria bacillus at one time or another during the yearly period.

There is, it appears, ample opportunity for contact with virulent diphtheria bacilli, and there is every reason to suppose that the increasing proportion of Schick-negatives in the progressively higher age groups is a consequence of an active immune response to the presence of these microorganisms in the

nose and throat.

The control of diphtheria. It will be obvious from the above considerations that diphtheria is widely disseminated in the human population and cannot be controlled by the isolation of carriers or, except in a strictly limited sense, by quarantine of cases. The diphtheria bacillus is especially sensitive to erythromycin, which has been used with some success in treating carriers. 6, 50

The control of diphtheria is a matter of immunization and, if a sufficiently large proportion of the susceptible population is rendered immune, the prevalence of clinical diphtheria should decrease. Godfrey found that the immunization of 50 per cent or more of the children of school age, five to 14 years of age, did not produce a fall in the incidence of diphtheria in a number of large American cities, but that when 30 per cent or over of the preschool children were immunized there was a definite reduction in the incidence of diphtheria not only among these children but in the community as a whole. It is commonly assumed that immunization of 70 per cent of children suffices to control epidemic diphtheria. Other as yet unknown factors are involved also, for the disease persists in relatively high incidence in some well-immunized communities or groups. 30, 33

To what extent prophylactic inoculation and the therapeutic use of antitoxin have influenced the decline in diphtheria is problematical. It is believed by some that the present decline is in part a continuation of a periodic trend, accelerating in the past two decades.^{34, 47}

The disease was endemic during the first

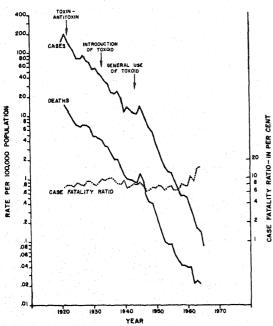


Figure 176. The decreasing prevalence of diphtheria in the United States during the period 1920–1965 as indicated by the morbidity and mortality rates. Note the lack of effect of the introduction of toxoid on the general trend, and the transitory rise during World War II. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

half of the nineteenth century though showing increasing epidemic tendencies. Between 1850 and 1860 a great pandemic developed, apparently from a focus in France, which swept over the world. A high mortality was maintained for 25 to 30 years, then around 1885 a decline set in which continued to about 1941. Immunization was not generally practiced until about 1920, though antitoxin therapy began somewhat earlier.

Beginning in 1941 there was a general increase all over the world in the prevalence of diphtheria. It apparently began in Germany in 1939, possibly due in part to mass movement of children into camps without adequate immunization, with a doubling of the already high (285 and 207 in Austria and Germany) morbidity rate, and an increase in severity as indicated by the increase in case fatality rates of 3.8 per cent in 1937–38, to 4.4 per cent in 1939, and 5.0 per cent in 1940. The disease spread into neighboring countries in northwest Europe. In Belgium the number of cases rose from 2419 in 1939 to 16,072 in 1943; in Holland

there were 1273 cases in 1939, 5501 in 1941, 19,527 in 1942, and 56,603 in 1943; in Norway there were 54 cases in 1939 and 22,787 in 1943. Diphtheria was the leading epidemic disease of the war years in Europe and an important cause of death in the German army. The rates have subsided from these highs, but diphtheria continues to be more prevalent in Europe than elsewhere in the world.⁴¹

These rates were reflected in U.S. military personnel stationed in Europe; diphtheria was a major problem with an incidence six times greater than for comparable troops stationed in the United States. Since 1946 the rate has declined. The number of cases and deaths in 1945 exceeded those of the previous five years, but the case fatality rate was not appreciably altered.³¹ In the United States there was a slight rise in the incidence of diphtheria, but it has subsequently declined; in 1966, 204 cases were reported in contrast to 17,987 in 1941. The decline has been in infants and children for the most part, but since 1940 there has been a slow but steady increase in the age groups over age 10,48 possibly a reflection of failure to re-immunize in the higher age groups. The residual infection tends to be concentrated in the nonwhite segment of the population. and further reduction would appear to be, in part, a matter of immunization of this group.35

THE DIPHTHEROID BACILLI

Microorganisms morphologically closely similar to and frequently indistinguishable from the diphtheria bacillus, known as diphtheroid bacilli, are found in man and lower animals. Three species are commonly observed in man. A form occurring in the human throat and readily confused with the diphtheria bacillus on microscopic examination was first observed by Löffler and by von Hofmann-Wellenhof. It is known as Hofmann's bacillus, Corynebacterium hofmannii, or C. pseudodiphtheriticum. It differs slightly from the diphtheria bacillus in that it is somewhat shorter and plumper and does not ferment dextrose. Most important, it does not form a soluble toxin and is readily differentiable from C. diphtheriae by the virulence test. It seems to be completely nonpathogenic for man and experimental animals.

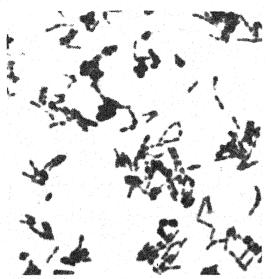


Figure 177. Corynebacterium pseudodiphtheriticum. Smear from pure culture stained with alkaline methylene blue. Note the irregular staining, club-shaped forms, and general close resemblance to C. diphtheriae. × 2000.

A second species, C. xerose, has been isolated repeatedly from a form of conjunctivitis known as xerosis, but its etiological relation to the disease is uncertain. It is also found on the skin, where it is presumably a part of the normal bacterial flora, and it is probable that its presence in xerosis is that of a contaminant. It does not form a soluble toxin. C. acnes has been found in acne pustules, but whether the association is causal is open to question. This organism stands somewhat apart from the other bacilli of the group in that it is micro-aerophilic and grows profusely under anaerobic conditions with the formation of a pink pigment. It also does not form a soluble toxin.

Animal pathogens. A number of species of corynebacteria are pathogenic for lower animals and rarely may infect man. C. pyogenes is one of the commonest causes of purulent infections in cattle, sheep, pigs, and goats; it is the cause of a form of mastitis, a few cases of abortion, arthritis, and granulomatous lesions in bovines, and is associated with calf pneumonia as well. Infections of man have been reported.3 It forms a soluble toxin, immunologically distinct from and considerably weaker than that of the diphtheria bacillus, which is hemolytic for rabbit erythrocytes and lethal for mice, and produces dermal necrosis in the rabbit similar to that produced by diphtheria bacillus toxin. This bacterium and its toxin have been studied extensively by Lovell.

C. ovis (C. pseudotuberculosis) or the Preisz-Nocard bacillus is also a pathogen of domestic animals. It produces a caseous lymphadenitis and ulcerative lymphangitis in sheep and horses referred to as pseudotuberculosis, and ulcerative lesions in other domestic animals. Like C. pyogenes, it forms a weak exotoxin distinct from diphtheria toxin. C. renale is closely related serologically to C. ovis and produces purulent infections of the urinary tract in cattle, sheep, horses, and dogs. C. equi is the cause of spontaneous pneumonia in foals and other infections in horses; this species is of interest in that it is variable in its reaction to the acid-fast stain, the coccoid forms retaining the stain while the bacillary forms take the counterstain, suggesting a relationship to the mycobacteria and acid-fast actinomycetes. C. enzymicum has been isolated from man primarily, but has been found as the cause of an epidemic ophthalmia of sheep. C. murisepticum is the cause of mouse septicemia and is apparently pathogenic for no other animals. The occurrence of these and other diphtheroid bacilli in diseases of domestic animals makes this group of microorganisms of considerable interest in veterinary medicine.

In addition to these a dozen or more authenticated species of corynebacteria are soil saprophytes or pathogenic for plants, producing diseases of wheat, alfalfa, ring rot of potato, bacterial canker of tomato and poinsettia, bean wilt, and the like.

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Chapter Thirty-one

MYCOBACTERIUM

This genus includes a number of species of related bacteria which are most conveniently considered in three groups. The first includes the mammalian tubercle bacilli Mycobaterium tuberculosis var. hominis and Myco. tuberculosis var. bovis, and the avian tubercle bacillus, Myco. avium, together with the so-called "anonymous" or "atypical" mycobacteria. In the second group there are Hansen's bacillus or Myco. leprae and the rat leprosy bacillus, Myco. leprae murium. The third group is made up of Johne's bacillus, or Myco. paratuberculosis, and certain acid-fast bacilli isolated from cold-blooded animals, together with the saprophytic acid-fast forms. 11

THE TUBERCLE BACILLI

Tuberculosis is an old disease of man and is still one of the most widespread; it is estimated that over a million persons in the United States have active tuberculosis, and about half of these are known to the health departments. Its infectious nature was suspected by Fracastorius in the early part of the sixteenth century, and Villemin showed, in 1865, that the disease could be transmitted by the inoculation of tuberculous material. It was in 1882 that Koch demonstrated the tubercle bacillus by special staining methods, isolated and grew it in pure culture, and reproduced the disease by the inoculation of the bacilli.

Morphology and staining.36 The tubercle bacilli are slender, sometimes slightly curved, rods 2 to 4 μ in length and 0.3 to 1.5 μ in breadth. They occur singly but are often found in small groups, sometimes in compact masses in which the individual bacilli cannot be distinguished. The bacilli of the human

variety tend to be somewhat longer and more slender than those of the bovine type, but the morphology of both is variable, and no distinction can be made on this basis. The bacillary form is generally retained in the tissues; in culture longer filamentous forms are sometimes seen together with swollen or club-shaped cells resembling the diphtheria bacillus. Branched forms are present in cultures of the avian tubercle bacillus but are rarely seen in cultures of the mammalian bacilli. The occurrence of filamentous forms and true branching indicates the close relation of these bacilli to the higher fungi.

The tubercle bacillus is nonmotile and nonspore-forming and produces a capsular substance in artificial cultures, particularly when grown upon serum mediums. The granular structure of the individual cells is marked. Vacuoles often occur in abundance and may even give the stained cell the appearance of a chain of cocci. The significance of the small, deeply staining bodies sometimes observed within the cells is not clear; they do not show the enhanced resistance

characteristic of spores.

The tubercle bacilli cannot be stained by the usual staining methods that are effective with other bacteria, for there is a marked resistance to the penetration of dyes into the cell that is associated with the presence of relatively large amounts of unsaponifiable wax. The cells may be stained in two or three minutes by steaming carbol-fuchsin or by 18 hours' exposure to the dye at room temperature. Once stained, the bacilli are difficult to decolorize and resist the action of alcohol and dilute solutions of mineral acids and for that reason are termed "acid-fast." Retention of the fuchsin is considered to be, in part, a matter of permeability. 62 They may be demonstrated in smears by the Ziehl-Neelsen method, in which the smear is stained with hot carbol-fuchsin, declorized with acid alcohol, and counterstained with a dye of contrasting color. Methylene blue is most commonly used but some workers prefer other stains such as picric acid and Bismarck brown. Other stains, Sudan black B and neutral red, have been used for experimental purposes. It has been reported that bacilli from the lungs of infected mice are stained only by the former, and those grown in culture only the latter.117 The bacilli may also be stained with carbol-auramine, a dve that fluoresces a brilliant yellow in weak ultraviolet light. This method is seldom used, and then only for screening purposes. Nonacid-fast bacilli may be observed in young cultures.

Nonacid-fast but gram-positive granules, known as Much granules, were described by Much in 1907 as occurring in the material from cold abscesses and elsewhere in which acid-fast bacilli could not be demonstrated but which, nevertheless, proved to be infective. Considerable numbers of acid-fast bacilli, perhaps 100,000 per ml., must be present, however, before there is a reasonable chance of finding them in smears. Much maintained that these granules are viable and virulent and give rise to typical acid-fast rods. They have been observed by others, but their significance is open to question. Some regard them as degeneration products or artifacts of the staining procedure.

In broth cultures there is a thick, wrinkled

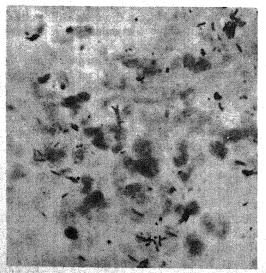


Figure 178. Mycobacterium tuberculosis. Acid-fast stained smear to tuberculous sputum. × 1050.

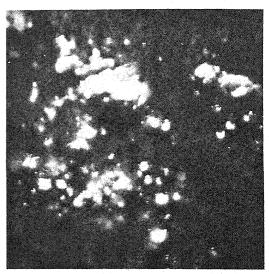


Figure 179. Colonies of the human variety of the tubercle bacillus, H-37 strain, on Löenstein's medium, at five weeks' incubation. \times 5.

skin of surface growth which tends to spread up the sides of the flask; masses of bacilli may become detached and sink to the bottom as a lumpy sediment. Growth on the surface of solid mediums is generally dry and granular with nodular, heaped-up areas. The human variety of the tubercle bacillus usually produces a pale yellow or orangeyellow growth on serum-containing mediums and a creamy or white growth in the absence of serum. The bovine variety is not pigmented on serum mediums. Some avian strains give a faint pink-colored growth on egg mediums. A peculiar almond-like odor is often noticeable in cultures of these bacteria.

Physiology. The tubercle bacillus is an aerobe and will not grow under completely anaerobic conditions. The mammalian varieties grow best at 37° C. and not at all below 30° C. or above 42° C.; the optimum temperature for the avian type, however, is 40° C. Growth is relatively slow, and four to six weeks are generally required for an abundant growth, although minute colonies appear in eight to 10 days. Most strains of the avian type adapt themselves readily to culture on artificial mediums and in time are able to grow much more rapidly, but others remain slow-growing.

Enriched mediums are required for primary isolation, and these usually contain eggs, glycerol, and sometimes dyes to inhibit the growth of contaminants. The commonly

used mediums are the Jensen modifications of Löwenstein's medium, containing egg, potato meal, bone marrow infusion, citrate, glycerol and asparagin, and malachite green; the egg yolk-potato medium of the American Trudeau Society; and McNabb's or Frobisher's modification of Petragnani's medium. Glycerolated potato is used by the French workers. Corper's medium is glycerolated potato with the modification that the pieces of potato are soaked for a short time in a solution of crystal violet before sterilization with the glycerol solution.

The human variety of the tubercle bacillus grows more abundantly on these mediums than does the bovine variety, and for that reason it is termed "eugonic" and the bovine type "dysgonic." These two varieties also differ in that glycerol is markedly favorable to the growth of the human type but does not so affect the bovine type. It is not known why glycerol exerts this favorable effect; attempts to substitute related compounds such as isopropyl alcohol, propyl alcohol, glycol, trimethylene glycol, and inositol have not been successful. Glucose, however, acts in much the same manner as glycerol. The metabolism of a variety of carbon compounds, including those functional in the Krebs cycle, has been studied in detail by Youmans and Youmans. 162 Egg yolk has been reported to contain a lipid growth factor, but this appears to stimulate rather than be essential to growth. Thiamin, pyridoxin, and riboflavin do not stimulate growth.

Growth occurs much more readily and upon simpler mediums after primary isolation. The human tubercle bacillus grows well upon nutrient agar or broth containing glycerol (2 to 5 per cent) and has been cultivated in a variety of synthetic solutions. One of the best known of these is Long's synthetic medium, which contains glycerol, asparagin, citrate, and inorganic salts. Dubos and his co-workers42 have found that growth is facilitated and occurs diffusely throughout the liquid medium in the presence of certain water-soluble lipids. A medium containing asparagin, glucose, phosphate, citrate, bovine serum albumin, magnesium sulfate, and a lipid commercially designated Tween 80 (a polyoxyethylene derivative of sorbitan mono-oleate), though it has appreciable growth-inhibiting activity, will give visible growth of the human variety of tubercle bacillus within two weeks. The medium becomes inhibitory in time, owing to the activity of lipase-contaminated bovine albumin preparations, which liberates oleic acid to bacteriostatic concentrations from the Tween 80; the effect can be eliminated by the use of commercial crystalline bovine serum albumin, by the addition of 0.01 per cent sodium fluoride, and by other means. The bovine type grows poorly or not at all on these mediums and is benefited only slightly by the presence of glycerol. The avian type of tubercle bacillus, however, grows better than the human type after a few transfers, and good growth may be obtained on nutrient agar in the absence of glycerol. Like the human type the avian bacillus grows much more profusely in the presence of glycerol. These cultural differences in the three types of tubercle bacilli are of little significance in their early differentiation, for several transfers over a period of months are necessary before they are obvious.

The biochemical reactions of the tubercle bacilli have not been studied at length. Growth occurs in milk, but no visible change is produced. Indol is said not to be formed. In glycerol broth, cultures of the bovine type become alkaline, while those of the human type become slightly acid. The conventional fermentation test is not readily applicable to the tubercle bacilli because of their very slow growth. The necessary prolonged incubation tends to give inconsistent results, possibly because of further decomposition of organic acids, concurrent deamination, etc. The use of very heavy, nongrowing suspensions of the bacilli in carbohydrate-containing mediums has indicated the possibility of useful differentiation. It is reported¹⁴⁰ that strains pathogenic for man but not for chickens ferment trehalose but not xylose, while strains pathogenic for chickens but not for man ferment xylose but not trehalose. The reduction of nitrate and the decomposition of nitrite have also been suggested as differential characters. 156

Growth in cell culture. The growth of tubercle and related bacilli in animal tissue cells in culture has been of interest since the early studies of Maximov on the pathogenesis of such infections. More recently cultures of spleen and liver explants, monocytes, and cells of the HeLa line have been used as substrates with particular reference to the differentiation of strains with respect to virulence. Infection of the host cells in maintenance mediums occurs within the first day or so, and proceeds rapidly in

growth mediums. It has been observed consistently that virulent strains grow more rapidly, with appreciable growth in three to five days, and there is concurrent destruction of the host cells. The tissue culture method appears to be less sensitive than conventional culture for primary isolation.

Chemical composition. The chemical composition of the tubercle bacilli has been more intensively investigated than that of any other bacteria. These bacilli are of particular interest in this connection because of their high content of lipoidal substances, which may make up as much as 40 per cent of the dry weight. Protein, a considerable proportion of which is nucleoprotein, makes up about half the dry weight, and polysaccharides are found in relatively small amounts. 119

The lipids have been studied at length by Anderson¹ and his co-workers. In addition to neutral fat, two general types of material may be distinguished:

(1) Phospholipid, containing saturated and unsaturated fatty acids including the well known palmitic, linoleic, and linolenic acids, together with two acids peculiar to the tubercle bacillus, *phthoic acid*, isomeric with cerotic acid, and *tuberculostearic acid*, isomeric with stearic acid and optically inactive.

(2) An acid-fast wax, containing polysaccharides hydrolyzing to mannose, arabinose, and galactose; a soft wax which is a complex glyceride; and an unsaponifiable wax (acid-fast) made up of higher alcohols and including a high molecular weight saturated hydroxy-methoxy acid termed mycolic acid; a higher alcohol designated phthiocerol; and a levorotatory fatty acid, mycocerosic acid. Acid-fastness is associated with the presence of mycolic acid.

Some of these substances appear to be physiologically active. The unsaponifiable acid-fast wax apparently stimulates the multiplication of undifferentiated connective tissue cells, and phthoic acid induces a proliferation of epithelioid cells. 113 A toxic lipid fraction, extractable in monochlorbenzene. is thought to contribute to the primary toxicity of massive inoculums, but is of uncertain significance in the disease. 135 A yellow pigment is found in the neutral fat which is designated phthiocol and is a hydroxynaphthaguinone which may be reversibly reduced at a relatively low potential. This substance is identical with vitamin K except for the substitution of a hydroxyl group for the phytyl radical on the third carbon.

Polysaccharide mixtures, containing immunologically active and inactive substances, have been isolated from mammalian tubercle bacilli, but their significance is not as yet understood. The protein constituents of the cell appear to be the most important immunologically and have been studied in connection with the preparation and activity of the various tuberculins.

Cord factor.¹² The tendency of tubercle bacilli to grow in filaments, or cords, in liquid culture was found by Middlebrook, Dubos, and Pierce in 1947 to be associated with the virulence of human and bovine varieties of the microorganism. On treatment with petroleum ether, the filaments break up, and a lipid substance is extracted which is acid-fast and toxic to leucocytes and mice. This substance was designated the cord factor by Bloch. Its association with virulence is not altogether clear, for it may be extracted from the attenuated BCG strain (see below) and from nonpathogenic forms such as the smegma bacillus.

Resistance. Although having much the same degree of resistance to heat as the vegetative cells of other bacteria, the tubercle bacilli are relatively highly resistant to drying, chemical disinfectants, and other deleterious environmental influences, very likely as a consequence of their contents of wax. In putrefying sputum the bacilli may remain viable for weeks or months and in dried sputum kept in a cool dark place for as long as six to eight months. Sputum that is completely dried, so that particles are capable of floating as dust in the air, may be infective for eight to 10 days. In dried sputum they may survive 100° C. for an hour but are killed in the usual way by moist heat. Phenol penetrates the bacilli only slowly, and a 5 per cent solution requires 24 hours to kill the bacilli in sputum. The action of other disinfectants is similarly retarded, and hypochlorites and certain synthetic detergents have almost no effect on these bacteria. Tubercle bacilli are readily killed by exposure to direct sunlight; bacilli from cultures are killed within two hours but in sputum may survive 20 to 30 hours of such direct exposure.

A number of different kinds of substances are bacteriostatic for the tubercle bacilli, including streptomycin, ρ -aminosalicylic

acid (PAS), sulfones, thiosemicarbazones, and certain pyrimidine derivatives such as isoniazid. These substances are extremely useful for chemotherapeutic purposes (see below).

Variation. The variability of the tubercle bacilli has been studied extensively, but with inconclusive results. Colonial variants. thought by some to be analogous to the S and R variants of other bacteria, have been observed, and it has been claimed by some workers that the S type is the more virulent. and by others that virulence and colonial morphology are independent. The colonial morphology of the tubercle bacilli is, to a considerable degree, a transient adaptation to environmental conditions, and prompt alteration of colonial appearance results on transfer to a different medium. For example, it has been observed that colonies growing in the presence of ether extract of egg volk are smooth and markedly different from the usual colonial type, but the effect is only a temporary physical one. The status of the S-R variation in the tubercle bacilli is, then, by no means clear as vet.48

Bacille Calmette-Guérin. A bovine strain of the tubercle bacillus was rendered completely avirulent by Calmette, who cultivated it over a long period (230 transfers in 13 years) on bile-glycerol-potato medium. This strain is designated as BCG (Bacille Calmette-Guérin) and has been of particular interest in connection with active immunization against tuberculosis. The nature of the change which resulted in the loss of virulence is completely unknown. The loss appears to be permanent and virulence does not reappear on transfer to ordinary mediums.

Life cycles. The pleomorphic tendencies of the tubercle bacilli, coupled with the occurrence of nonacid-fast rods in young cultures and the granular elements described by Much, have been interpreted by a number of workers as indicative of a cyclic succession of morphological types, or life cycle, through which these bacilli go. As pointed out elsewhere (Chap. Seven), evidence suggesting the occurrence of life cycles of bacteria is subject to various interpretations.

Filterable forms. L variation⁷⁸ occurs during which viable granules, perhaps to be regarded as Much granules, are produced. The viable fragments, including filaments as well as granules, revert to the bacillary

form on culture on the conventional mediums. The latter probably account for the many reports of filterable forms, or "ultravirus" of tubercle bacilli.

Drug resistance. Like other bacteria, the tubercle bacilli may become resistant to chemotherapeutic drugs in vitro and in vivo.2, 22, 99, 121 The latter is more common with these bacteria than with other kinds because of the nature of the disease and the prolonged treatment required, and because resistance to certain of the effective drugs, streptomycin and isoniazid in particular, is especially prone to occur. Isoniazid resistance is accompanied by a loss of catalase activity by tubercle bacilli, but some catalase activity persists in resistant saprophytic mycobacteria. There is evidence that the latter contain two catalase systems, one of which is not isoniazid-sensitive.145

The practical importance of drug resistance depends upon the drug and its toxicity. For example, a blood level of 10 to 15 μ g. per ml. of streptomycin may be attained, and sensitive strains are inhibited in vitro by 0.5 μg. per ml., slightly resistant strains by 2 to 4 µg. per ml., moderately resistant by 200 to 400 μ g, per ml.; and more than 50,000 μ g. per ml. is required to inhibit the growth of highly resistant strains. Blood levels of isoniazid of 3 μ g. per ml. may be attained, and sensitive strains are inhibited by 0.025 μg. per ml. in vitro. Thus a proportionately higher resistance must occur in the case of isoniazid than of streptomycin to allow growth in therapeutic concentrations. The assay of drug sensitivity is complicated by the slow growth of the bacilli17, 139 and more rapid methods have been devised based on the use of suspensions of bacilli and indicators of oxidative metabolism such as resazurin or tetrazolium. 69, 75, 148

The facility with which resistance to a single chemotherapeutic agent develops is such that for practical purposes it is necessary to reduce the development of drug resistance by the use of combinations of drugs. As described elsewhere (Chap. Seven), if the resistant strain arises as a consequence of the selection of a chance mutant by the drug-containing environment, and resistance to one drug is independent of that to another, the probability of occurrence of a double mutant is given by the product of the two mutation rates and becomes extremely small. The development of drug resistance in vivo

is, in fact, markedly inhibited by the use of two drugs in combination, commonly streptomycin plus PAS, isoniazid plus PAS, etc. Resistant strains do not appear to differ significantly from the sensitive parent strain except in the case of those resistant to isoniazid, which are appreciably less virulent though still capable of producing fatal disease in man. 138, 144

Pathogenicity for man. Tuberculosis is still the most important specific communicable disease in the world, affecting more than 50,000,000 people. In the United States 50,000 new cases are reported each year, and there are about 10,000 deaths annually. It is estimated that of 30,000,000 people currently infected but without evidence of active infection, some 2,000,000 will eventually develop tuberculosis.

The mammalian tubercle bacilli, both bovine and human varieties, are pathogenic for man. The human type is practically always responsible for pulmonary tuberculosis in adults and is usually found in children also. The bovine variety may occur occasionally in pulmonary tuberculosis in children but is more often found in infections of other tissues. Mixed infections with the two types of tubercle bacilli have been reported but are rare.

The avian tubercle bacillus is generally considered to be, for all practical purposes, nonpathogenic for man. Cases of human infection, however, are reported from time to time, ^{23, 43, 76} and some studies have suggested that it may be more important in human disease than generally believed. In one study carried out in Germany, 8.5 per cent of 218 positive specimens were avian tubercle bacilli. ⁹¹ It resembles certain of the anonymous mycobacteria (see below), with which it may be confused.

Routes of infection. The tubercle bacillus may enter the body by way of the genitourinary tract, the conjunctiva, the skin, the alimentary tract, and the respiratory tract. Primary infection of the genitourinary tract is possible but, under natural conditions, rarely occurs. Infection through the conjunctiva takes place readily under experimental conditions; its frequency under natural conditions is not known, for the cervical lymph glands, where the infection would first appear, are readily infected by other channels. Infection through the skin is relatively rare; whether the bacilli can penetrate the intact skin is uncertain, but

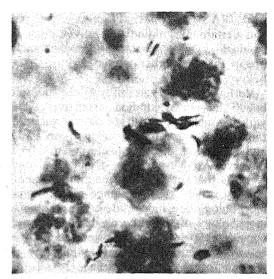


Figure 180. Tubercle bacillus. Acid-fast stained smear of pus from a liver abscess in a rhesus monkey. × 2600.

they may enter through abrasions or other traumatic injuries. Primary infection of the skin generally results in verruca tuberculosa (pathologist's wart) or lupus vulgaris.

Primary infection by the alimentary tract is a consequence of the ingestion of tubercle bacilli in infected food, most commonly milk, and occurs with considerable frequency in children. Secondary infection may occur, especially in children, by swallowing tuberculous material of respiratory origin. In the upper portion of the alimentary tract the bacilli enter the body tissues through the faucial, pharyngeal, and lingual lymphoid follicles and first affect the upper cervical and retropharyngeal lymph glands. Tuberculous lesions of the tonsils are not uncommon though rarely conspicuous. The stomach is rarely a portal of entry, but the bacilli penetrate the intestinal mucosa by way of Peyer's patches.

The respiratory tract is the most frequent and most important route of infection with the tubercle bacillus, and the facility with which this occurs is demonstrable under both controlled and natural¹¹¹ conditions. The coarser infectious particles in inspired air are filtered out and deposited on the nasal, buccal, and pharyngeal surfaces, and the bacilli, upon penetration, set up focal infections in the local lymphatic tissue. Fine droplets or particles of dust, however, may, and frequently do, enter the lungs directly (Chap. Ten).

The spread of infection in the body. bercle bacilli are disseminated through the body from primary or secondary foci of infection by way of the lymph or the blood stream, or directly by extension along contiguous surfaces. Distribution via the lymphatics occurs more readily in children than in adults, and the bacilli may localize at almost any point but most commonly in the lymph nodes. Bacilli present in the thoracic duct may gain entrance to the blood stream. The blood stream may also be invaded directly through erosion of a vessel wall by a focus of infection. The bacilli are transported throughout the body by the blood and give rise to acute miliary or chronic disseminated tuberculosis. An example of the pathogenesis of hematogenous spread occurred following an instance of intravenous self-inoculation with suicidal intent:59 general miliary infiltration throughout both lungs occurred between the sixteenth and twenty-first days, but the tuberculin reaction did not become positive until the twentysixth day.

Spread by extension occurs most frequently in ulcerative pulmonary tuberculosis with the breaking down of foci of infection and the consequent presence of bacilli in the sputum. The pleura and pericardium may be invaded directly from a focus of infection in the lungs. The infection of such serous surfaces may be localized and fibrinous and result in adhesions, or it may assume an acute miliary form. Direct extension may also occur elsewhere, as from the kidneys to the ureters and bladder or into the peritoneal cavity and adjacent areas from intestinal ulcers. Practically every organ and tissue of the body may be invaded by the tubercle bacillus.

Tuberculosis in man is most commonly the pulmonary form with a primary invasion of the apices, and over 90 per cent of the deaths from tuberculosis are due to the pulmonary type. Whether primary infection is a direct consequence of inhalation as believed by Koch, or whether it is in most cases of hematogenous origin following a preliminary infection of the lymphatic system as urged by Calmette, is not altogether clear, but on the whole the evidence favors inhalation infection. Although pulmonary tuberculosis is by far the most common form of infection in adults, it is somewhat less frequent in children, but precisely how much less is not known with certainty. Pulmonary infection in children differs from that in adults in that the hilum glands are involved. Involvement of the lymphatics is frequent in children. The anatomical distribution of the lesions usually takes one or the other of two well-defined forms; in the one the lesions are found predominantly in the tracheobronchial lymphatics and in the other in the mesenteric lymph nodes. In general, children show a tendency toward generalized infection.

Other tissues and organs are involved less frequently. The spleen, liver, and kidneys are sometimes infected. Tuberculosis of the adrenals gives rise to Addison's disease. Infection of the skin or lupus is not uncommon. Bone and joint tuberculosis is more common in children than in adults, and tuberculosis meningitis is not infrequent in the young. It will be clear that tuberculous infection may take a variety of clinical forms.

The tubercle. Lesions caused by the tubercle bacillus, in whatever part of the body they occur, usually possess a definite although not absolutely characteristic appearance and histological structure. Small nodules or tubercles, plainly visible to the naked eye, are so uniformly observed in advanced infections with the tubercle bacillus that their presence has given the name to the disease. The young tubercle probably originates from the fixed cells surrounding the invading bacilli. By the proliferation of the fixed cells, elongated "epithelioid" cells are developed in more or less definite concentric layers and come to form the substance of the tubercle. So-called giant cells or foreign-body giant cells soon appear in the developing tubercle; these are huge multinuclear masses of protoplasm thought by some to be especially distinctive of true tubercle formation, though it is doubtful if this criterion can be maintained. Either they are produced by the fusion of a number of macrophages or they are of single-cell origin. While the formation of epithelioid and giant cells is going on, leucocytes, at first polymorphonuclear leucocytes and later lymphocytes, cluster around the periphery of the tubercle. Degeneration of the tubercle eventually sets in, the central portion becomes necrotic, and this is followed by caseation and then by softening of the caseous mass.

In some cases calcium salts are deposited in the tubercle (calcification), converting it into a hard, dry, friable body which may become encapsulated and completely walled off from the surrounding tissues. In other instances, however, this healing process does not take place, and there is instead an extension with coalescence and the formation of confluent masses that may reach a diameter of 4 or 5 cm. The erosion of a blood vessel may occur, and the discharge of large numbers of the bacilli into the blood stream leads to a general diffusion of small tubercles of the size of millet seeds (acute miliary tuberculosis).

The early stages of tubercle formation, characterized by cell proliferation and leucocytic infiltration, are probably a response to a chemical or mechanical stimulus caused by the presence of the bacilli; the later changes, leading to necrosis and caseation, may be attributed to the action of the bacterial products. The host becomes sensitized to the bacillary substance, and the allergic response of the tissues is involved to no small extent.

Predisposing factors. Few diseases are as completely dominated by predisposing factors as tuberculosis. Infection with the tubercle bacillus is exceedingly common, and there are few adults, particularly those living in cities, who escape. The proportion of individuals coming to autopsy who show evidence of infection is very high and has been reported as high as 97 per cent. In one series of cases, for example, it was found that 25 per cent of 10-year-olds, 55 per cent of 20-year-olds, 80 per cent of 30-year-olds, and over 90 per cent of individuals over 50 years of age show calcified lesions of infection. The bacilli, then, are present in the great majority of adults and also in many children, but the development of clinical tuberculosis is restrained by the nonspecific resistance of the host. For example, in one study of tuberculin-positive children carried out prior to the advent of chemotherapy, 91 per cent showed no clinical illness though radiologic signs were present but eventually resolved, 5.4 per cent had clinical illness but recovered, and 3.6 per cent died.90

Predisposing factors, therefore, are those which tend to interfere with normal physiological well-being and include such things as insufficient or unsuitable food, prolonged exposure to dampness, a sedentary life, and chronic fatigue. In World War I the incidence of tuberculosis rose in this and other countries, and in World War II the same

tendency was again apparent in Europe. In both man and animals tuberculosis is a disease associated with confinement, of men in houses and cattle in stables. The opportunities for transmission of the infection are, of course, much greater in community life.

There is a marked occupational predisposition to tuberculosis in the dusty trades, and the constant inhalation of almost any kind of dust results in an increased incidence of pulmonary tuberculosis. Silica dusts, however, appear almost specifically to predispose to tuberculosis, and the incidence of infection in those constantly exposed to such dusts is much higher than that in the general population.

It has long been suspected that there are familial or hereditary tendencies to tuberculosis in man. Resistance to infection in experimental animals is to some degree genetically determined, but the conclusive demonstration of a similar phenomenon in man is difficult owing to both a long generation time and the inability to carry out appropriate breeding experiments. The incidence of new infection in tuberculous families is considerably greater than in the general population, but whether this is a consequence of increased risk alone or, in part, of genetically determined factors is not clear. In any case the disease itself is not inherited. and congenital tuberculosis occurs only rarely.57, 155

The relation of childhood infection to clinical tuberculosis in the adult has been a matter of considerable interest, and it has been suggested that pulmonary tuberculosis of the young adult may, in many instances, be a consequence of the lighting up of an old infection.²⁹ Tuberculosis in the adult may also be a consequence of reinfection rather than of a flaring up of the healed or partially healed lesions from childhood infection. A considerable period, possibly years in some instances, may elapse between reinfection and the appearance of tuberculosis in clinical form.

Bacteriological diagnosis.^{84, 102, 106} Tubercle bacilli are discharged from the infected individual in sputum and urine and may be demonstrated in these materials and in gastric washings, spinal fluid, or infected tissues, depending on the location of the infection. Of specimens from these sources, those obtained by gastric lavage are of particular value in respiratory tuber-

culosis in children, who tend to swallow sputum, and are also useful in adult infections. Results must be interpreted with caution because of the presence of saprophytic acid-fast bacilli in the gastrointestinal tract derived from the ingestion of foods, especially fruit, on which these forms occur; furthermore, there is some reason to believe that some fats and oils may impart acid-fast properties to ordinarily nonacid-fast bacteria present in the alimentary tract. The presence of the bacilli may be shown either directly or after concentration by examination of stained smears, 114 culture, and guinea pig inoculation.

As a rule, some method of concentration is desirable,³⁴ for not less than 100,000 bacilli per ml. must be present before there is a reasonable chance of finding them by microscopic examination. The resistance of the tubercle bacillus allows treatment of the specimen with hypochlorite or NaOH to destroy contaminants and digest the viscous sputum to facilitate concentration by centrifugation. The sediment is used to prepare smears for direct examination and to inoculate culture mediums and/or guinea pigs.

Smears are usually stained by the Ziehl-Neelsen method. The demonstration of acid-fast bacilli allows a provisional diagnosis but does not indicate whether the bacilli were viable or whether they are virulent tubercle bacilli. Their presence in urine specimens in particular must be interpreted with caution because of the frequent occurrence of the smegma bacillus, which is also acid-fast.

Guinea pigs are inoculated in the groin or in the muscle of the thigh. If a reasonable number of tubercle bacilli have been inoculated, enlargement of the regional lymphatics may be noted in two or three weeks, the pig becomes emaciated by four to six weeks and usually dies not long after. With very small numbers of bacilli evidence of the infection may be delayed two or three weeks longer. If it is not obviously ill or does not die, the animal should be kept for eight weeks before sacrificing. At autopsy the regional lymph nodes will be found to be enlarged and filled with caseous material. Necrotic areas in the spleen and liver are characteristic of the gross pathology in this animal; tubercles are seldom seen, and the lungs are only slightly affected and the kidneys almost never. Tubercle bacilli may be cultured from the lesions and found in acidfast-stained smears. Some workers tuberculin-test the animals before inoculation and three to four weeks afterward, the development of hypersensitivity indicating infection. If differentiation of the human and bovine varieties of the bacillus is desired, rabbits may be inoculated.

A variety of mediums, containing glycerol and egg, may be used for culture, the particular medium varying from one laboratory to another. The culture is commonly enclosed, as in a screw-cap culture tube or with paraffin poured over the cotton plug, to minimize drying, but a small hole needs to be left to allow air exchange. Characteristic colonies of tubercle bacilli appear after about three weeks' incubation. Extended incubation, to as long as five months, may give a few additional positive cultures. 103 Culture is not identification, of course, and acid-fast bacilli, which grow like the tubercle bacilli, are occasionally found in nontuberculous lesions.

Chemotherapy. 79, 80, 157 Effective otherapy of tuberculosis requires that the chemotherapeutic agent be diffusible into the tuberculous process as well as having specific antibacterial activity. In addition, the necessity for administration over relatively long periods accentuates the problems of toxicity and the development of drug resistance by the microorganism. Prior to 1938 no substances of practically significant activity were known, but while the ideal chemotherapeutic agent for this disease has not been found, a number of drugs of reasonable efficacy are now available. These fall into two groups, viz., the synthetic compounds and the antibiotics.

The synthetic tuberculostats. There are four groups of these: the sulfones, the aminohydroxybenzoic acids, the thiosemicarbazones, and the pyrimidine carboxylic acid derivatives. The observation that sulfanilamide affects the progress of experimental tuberculosis led to the preparation and trial of related compounds. Of these, the sulfones, 4.4'-diaminodiphenvl sulfone and its derivatives such as Promin, Diasone, and Sulphetrone, were found to be partially effective chemotherapeutic agents. All are toxic, the most common effect being on the erythrocytes, and, while toxicity may be reduced by adjusting dosage, it has not been possible to eliminate it, probably because the activity of derivatives is a consequence of their degradation in vivo to the parent compound.

The observation that benzoates and salicylates stimulate the respiration of the tubercle bacillus *in vitro* provided the basis for the discovery of the chemotherapeutic activity of *p*-aminosalicylic acid, the most active of the compounds tested. This substance is of low toxicity and is readily absorbed from the gastrointestinal tract. It has appreciable chemotherapeutic activity when given alone, but its greatest utility is in combination with streptomycin or isoniazid.

The thiosemicarbazones are more effective chemotherapeutic agents than the sulfones or salicylates and have been used relatively widely in Europe but not in this country. The compound p-acetaminobenzal-dehyde thiosemicarbazone (Tibione) has been tested extensively together with a number of derivatives. All of these are relatively toxic substances and give severe side reactions, including anemia and liver and kidney damage in addition to gastrointestinal disturbances.

A number of pyrimidine derivatives, isonicotinic acid hydrazide (isoniazid) and related compounds such as the isopropyl derivatives, are available under a number of proprietary names and are widely used and effective chemotherapeutic agents although they have some neurotoxicity.

Antibiotics.⁴⁷ Streptomycin was the first of the antibiotics to be used for the therapy of tuberculosis, and continues to be the most widely employed. Most other antibiotics effective in the treatment of acute infectious diseases are not satisfactory, but the broad-spectrum antibiotic cycloserine (D-4-amino-3-isoazolidine) has been used to a considerable extent as an effective chemotherapeutic agent.

As noted earlier, the various chemotherapeutic agents are commonly used in combination in the treatment of tuberculosis to minimize the occurrence of drug-resistant variants.^{27, 74} While commonly given separately, very many compounds or complexes of two drugs have been prepared under a variety of names, and are described elsewhere (Chap. Six).

Antibacterial activity in vivo. Assay of the chemotherapeutic efficiency of tuberculostatic drugs is dependent upon the nature and stage of the infection. For example, in early infection, growth of the bacilli is largely intracellular, and streptomycin and PAS do not penetrate macrophages, though isoniazid does, to affect the phagocystosed bacilli, but when the individual is allergic (see below), growth tends to be more frequently extracellular. Similarly, in tuberculous lesions the bacilli grow most densely at the periphery of the caseous zone, and as the lesion expands they are engulfed in caseous material and lie dormant. Such dormant bacilli are not appreciably affected by the bacteriostatic activity of drugs, and the concentraton of drug is lower in such material than in the blood. Thus a focus of infection may persist in the individual undergoing chemotherapy and result in relapse, and possibly infection with drug-resistant bacilli, when therapy is discontinued. The intracellular persistence of viable bacilli during chemotherapy may be of some small significance in pulmonary tuberculosis, but of very considerable importance in tuberculosis meningitis.

The anatomical changes occurring in the tuberculous lesion during chemotherapy have been summarized by Auerbach,3 who groups them as follows: (1) a rapid and extensive clearing of the perifocal reaction, (2) a greatly decreased width of fibrous capsules around necrotic foci, (3) decreased thickness of the cavity wall and overlying pleura, (4) a decrease in pulmonary fibrosis and emphysema, (5) a marked decrease in the occurrence of massive pulmonary hemorrhages, (6) a difference in the mode of cavity healing, and (7) an accelerated healing of the tuberculous process. The last is especially evident when treatment is begun early in the disease and appears as an accelerated development of collagen fibrils and a corresponding decrease of cells and capillaries of the granulation tissue around the necrotic foci and the walls of tuberculous cavities. The changes differ somewhat depending the chemotherapeutic upon agent.73

Immunity^{120, 151} The immune response of the animal body to the presence of the tubercle bacillus is indicated by the appearance of agglutinins, precipitins, opsonins, and complement-fixing antibodies in the serum. This response is not marked, however, for these antibodies are present only to low titers. Erythrocytes sensitized with tuberculin are passively agglutinated,⁸³ but this reaction appears to have only limited diagnostic or prognostic value.

The most striking immune response is the development of hypersensitivity of the delayed type to the bacillary cell substance. Within limits, this allergic response is protective against reinfection, as shown by Koch's early experiments. He showed that the subcutaneous inoculation of the normal guinea pig with tubercle bacilli produces no immediate response, but that in 10 to 14 days a nodule develops which breaks down to a persistent tuberculous ulcer, and the regional lymph glands become swollen and caseous. In the tuberculous animal, however, an indurated area appears within a day or two, and there is slight necrosis with the formation of a shallow ulcer which heals promptly without the development of gross tubercle tissue or the invasion of the adjacent lymphatics by the bacilli. This is known as the Koch phenomenon.

This resistance to reinfection is relative and is not shown unless the primary infection is of some weeks' standing or large numbers of bacilli are injected. Experimental studies with guinea pigs have shown that immunization increases the infective dose about 1000-fold.¹⁰ Animals may be sensitized not only by infection with virulent bacilli, but also by the inoculation of attenuated or killed bacilli. Sensitization with preparations of the bacillary cell substance is difficult and very large doses must be administered. Raffel¹⁰⁰ has shown that the reaction is a result of inoculation with tubercle bacillus protein combined with a purified wax fraction consisting of an ester of polysaccharide and higher alcohols with hydroxy fatty acids; the protein alone produces an immune response with formation of precipitins. A protein fraction separated by urea extraction has been found to sensitize animals,35 and an effective immunizing preparation of subcellular particulate material has been prepared from virulent human strains, and also from BCG but of much reduced immunizing efficiency, by Youmans and his co-workers. 163, 164, 169 Cell wall fractions alone have also been found to be immunogenic. 108, 109

The hypersensitivity so produced is of the delayed kind typical of infection allergies. It is not passively transferable by serum but may be transferred by cells from a hypersensitive animal.

Tuberculin. The sensitized animal will react to the soluble cell substance of the tubercle bacillus, preparations of which

have been called tuberculin. Tuberculin is usually prepared from the human type of tubercle bacillus, though bovine tuberculin is practically as active as human tuberculin in infections with the human bacillus; avian tuberculin, although considerably less active, will also produce a reaction. A variety of tuberculins have been prepared, of which only a few need be noted here. The first of these was made by Koch, and consisted of the filtrate of a glycerol-broth culture of the bacilli concentrated by evaporation on a water bath to about one-tenth its original volume (the activity is heat-stable). This material is "original" or "old" tuberculin (TO or OT). A "new" tuberculin (TR-tuberculin residuum) was prepared by Koch in 1897 by macerating living virulent bacilli, extracting the mass with water, and then making an emulsion of the residuum. He later advocated the use of an emulsion (BEbacillary emulsion or Bazillenemulsion) of the entire substance of young virulent bacilli in 20 per cent glycerol, actually a vaccine. Denys introduced the use of the unaltered filtrate from broth cultures (BF-broth filtrate). None of these later innovations, however, proved to be superior to old tuberculin, and the original preparation, or slight modifications of it, has been widely used.

The active principle of tuberculin is protein in nature, and the cultivation of the tubercle bacillus in glycerol-asparagincitrate synthetic solutions by Long and Seibert has made possible the study of the active principle in purified preparations. The activity is associated with a number of protein fractions, one of which Seibert prepared in crystalline form. A more satisfactory preparation of low molecular weight, ca. 2000, has been isolated by Seibert by precipitation with trichloracetic acid. Originally designated SOTT (synthetic medium old tuberculin trichloracetic acid precipitated), it is now known as PPD (purified protein derivative).118

The comparative merits of OT and PPD have been the subject of a series of investigations. OT is relatively unstable in dilutions, while PPD, a dry powder, is "dry-diluted" with lactose and is indefinitely stable in this form. Different lots of OT vary somewhat in their activity; the activity of PPD preparations is relatively constant. It appears that PPD is quite as satisfactory as OT in actual use and, because of its stability

and constant activity, is regarded by many as superior to OT.

The tuberculin reaction. 72 Three types of reaction may be elicited in the sensitized. i.e., infected, animal by the injection of tuberculin. In addition to a local inflammatory reaction at the site of inoculation, there is a focal reaction manifested as an acute congestion around tuberculous foci which, if marked, may aggravate the pathological process, and a constitutional reaction in which the temperature rises to a peak of 102° to 104° F. and subsides in 12 to 18 hours. In man the constitutional reaction also includes malaise, pain in the limbs, and, perhaps, vomiting, dyspnea, and other symptoms. These reactions do not appear in normal animals. The utility of tuberculin is, then, two-fold; it may be used for diagnostic purposes and it has therapeutic value, though the latter is strictly limited.

The diagnostic tuberculin test in man is generally a skin test. Koch's original method consisted of subcutaneous injection of tuberculin. The cutaneous reaction of von Pirquet involves the rubbing of tuberculin onto the scarified skin. In the Mantoux test, the one most commonly used today, graded doses of tuberculin are injected intradermally, usually starting with 0.01 mg. of OT and going as high as 1.0 mg. or even 10 mg. on rare occasions (0.1 ml. of a 1:100 dilution of OT is supposed to contain 1 mg.; the standardization of new batches is biological and carried out in guinea pigs infected with virulent tubercle bacilli). Smaller amounts of PPD are used, since it is in dry, pure form, usually 0.00005 to 0.005 mg. A "patch test" has been introduced by Vollmer in which squares (0.8 cm.) of thin filter paper, impregnated with tuberculin about four times as strong as the original old tuberculin and dried, are taped on the cleansed skin over the sternum or upper edge of the trapezius. The patch test appears to be somewhat less sensitive than the intracutaneous test of Mantoux. Since these are all skin tests, only the local inflammatory reaction is observed in infected persons. There is evidence, however, that repeated tuberculin testing may result in a local sensitivity in the uninfected per-SOn 41, 94

In young children a positive tuberculin reaction may be taken as indicative of infection. Reactivity may be temporarily depressed under some conditions, especially

in the incubation and early stages of measles, or by measles immunization. ¹³⁷ It was formerly believed that, once established, the hypersensitivity persisted essentially throughout life and that the tuberculin reaction was of limited value in the adult. It is becoming apparent, however, that reversion is more frequent than had been generally realized, particularly with reduction in the prevalence of the disease and therefore the risk of reinfection. ⁵⁸

The tuberculin test in cattle has great diagnostic importance and has been widely used in the United States and to a lesser extent elsewhere. Three types of test may be used in cattle: the intradermal test; the ophthalmic reaction of Calmette, in which tuberculin is dropped into the conjunctiva and the reactive animal responds with the development of a diffuse congestion and edema in six to eight hours which fades away in 24 to 36 hours; and the constitutional reaction as indicated by a rise in temperature following the injection of tuberculin. The intradermal inoculation into the skin of the caudal fold is generally practiced in this country.

The therapeutic value of tuberculin may be directly observed in lupus or tuberculous infection of the skin. As indicated above, there is a reaction about the foci of infection manifested as acute congestion and sloughing off of tissue. When it was first introduced, tuberculin was regarded by many as a highly effective specific therapeutic agent for tuberculosis. Its use is, however, exceedingly dangerous, and, with the exception of lupus, the response of tuberculous infection to the injection of tuberculin has been disappointing.

The mechanism of immunity. Infection with the tubercle bacillus confers a definite protection against reinfection, an "immunity to superinfection" resembling that observed in syphilis. The factors involved are as yet obscure. Some current opinion favors the view that the development of an allergic state is indicative of an effective immunity. It is commonly observed that in individuals giving a positive tuberculin reaction living cells are usually free of tubercle bacilli and the bacteria are present in necrotic areas separated by an avascular barrier, while in the infected individual giving a negative reaction, tubercle bacilli are found in great numbers in living tissue. The antibodies produced are apparently not

significant and antiserums have no protective or curative properties. As indicated above, the development of hypersensitivity is the most obvious response to infection, and there is no doubt that hypersensitivity plays a part in acquired resistance as indicated in Koch's early experiments. Its relative importance is, however, not at all clear, and the mechanism of what seems to be a low-grade immunity of short duration remains at the moment largely a matter of speculation.

Active immunization. The possibility of active immunization to tuberculosis has been of great interest since the discovery of the tubercle bacillus. In general, two types of vaccines have been employed, suspensions of living attenuated bacilli and suspensions of killed bacilli. 116

The attenuated bovine strain of Calmette, BCG, has been regarded as the most promising immunizing agent and has been extensively studied. It was originally given as an oral vaccine in France in the early 1920's. However, the combination of inadequate statistical data and an incident in Lübeck, Germany, in which a virulent strain was inadvertently substituted for the vaccine strain, resulting in tuberculosis in inoculated persons, put it into disrepute. Nevertheless immunization with BCG was further studied in the Scandinavian countries, beginning in 1925 in Sweden and in 1927 in Norway and Denmark. The freshly prepared vaccines have a relatively short. useful life, perhaps seven to 10 days, but lyophilized vaccines, stabilized by the addition of dextran146 or glutamate93, 158 after an initial loss of some viability, are stable for a year when stored at 20° C. or less. A heat-stable vaccine, stable for considerable periods without storage at refrigerator temperatures, has been developed.147 The vaccine is given intracutaneously in doses of 0.05 to 0.15 mg., and a positive tuberculin reaction appears in six to 10 weeks in over 90 per cent of those inoculated. The hypersensitivity lasts about four years, though in some persons there is reversion in as little as one year. On the assumption that a positive tuberculin reaction is indicative of immunity, reversion is taken to mean that reinoculation is required.

There is a large body of evidence^{9, 136} which supports the conclusion that such immunization gives an appreciable degree of protection against childhood tuberculosis,

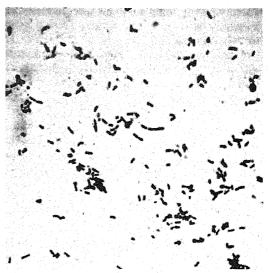


Figure 181. Avian tubercle bacillus. Acid-fast stain of a smear from a pure culture. × 1050.

but whether against infection later in life is not so clear. It is believed by some, 89 however, that the immunization, involving the use of a living vaccine whose virulence may not be fixed, is a dangerous one, and that confidence cannot be placed in immune response of the newborn and immature infant; fatal cases of infection with BCG have, in fact, occurred, 82, 152 but it is surprising that there have not been more in view of the many thousands of inoculations that have been carried out.

Immunization has been very generally applied in Scandinavian countries (where its effect on the decline in tuberculosis is probably overestimated), less so in Europe, and in only a restricted way in this country. While a general program of immunization may serve as a control measure in areas in which the disease is widespread and the general application of chemotherapy, isolation, etc., is not practical, 105 it appears to have only limited utility, as in groups exposed to unusual risk of infection, in this country. 46, 104

Pathogenicity for lower animals. Tuberculosis of lower animals living under natural conditions is probably very rare. Animals in captivity, however, may contract the infection with some facility; in animals kept in zoos and in monkeys kept in the laboratory for experimental purposes tuberculosis is not uncommon. Domestic animals may be similarly affected. A wide

variety of animals may be infected experimentally with one or another of the types of tubercle bacilli.

Domestic animals. The most commonly infected domestic animals are cattle, pigs, and chickens. Cattle are infected with the bovine type of tubercle bacillus almost exclusively; they are not completely resistant to the human type, as Koch originally thought, but infection is accomplished with some difficulty. The proportion of cattle infected increases with advancing age and, in the absence of control measures, may reach 70 to 90 and possibly 100 per cent in animals kept in stalls. The natural infection is generally of a chronic, slowly progressive nature. The lymphatics are most frequently involved and may be the only tissues to show lesions: the lungs are also commonly affected. Lesions on the pleura have a peculiar characteristic appearance, the so-called perlsucht disease. The liver, spleen, and kidneys are less frequently involved, infection of the mammary glands is not uncommon, and tubercle bacilli may be excreted in the milk in the absence of detectable lesions of the mammae. Congenital tuberculosis in cattle occurs with some frequency. In this country tuberculous infection in cattle is rigorously controlled; over nine million cattle are tested annually, and the percentage of infected cattle found is 0.19 and of infected herds 1.4 to 1.6.

Tuberculosis in chickens is very common and is exclusively an infection with the avian variety of the tubercle bacillus. With the exception of parrots and certain birds of prey, birds are highly resistant to infection with the human and bovine varieties, and it is probable that natural infection with these types rarely if ever occurs. Tuberculosis in chickens is usually a chronic process and is characterized by the formation of nodules in the abdominal viscera. The lungs are less frequently affected.

Pigs suffer from natural infection with both bovine and avian tubercle bacilli derived from infected cattle and poultry; they are also susceptible to infection with the human variety. In young pigs infection with bovine bacilli is generalized and acute with lesions in the lymphoid tissue, abdominal viscera, and lungs. Infection with the human bacillus is generally localized, but the avian type may produce a generalized infection.

Other domestic animals suffer from tuberculosis to a considerably lesser extent. Horses, dogs, and cats are occasionally infected, and the disease is rare in sheep

and goats.

Experimental animals. Experimental animals vary in their susceptibility to the varieties of the tubercle bacillus and in the type of infection produced. The guinea pig is highly susceptible to both bovine and human bacilli, and death follows the subcutaneous injection even of small doses in six to 15 weeks. The lymphatic glands, spleen, and liver are most affected, the lungs only slightly, and the kidneys never. The necrotic areas in the spleen and liver are the most striking feature of the gross pathology and are peculiar to the guinea pig. True tubercles are seldom seen except in the very early stages of the disease.

Rabbits are highly susceptible to infection with the bovine bacillus, somewhat so to the avian bacillus, and quite resistant to the human variety. Injection of bovine bacilli produces a generalized infection that terminates fatally in two to three months. On autopsy tubercles may be found in the spleen and liver, but the lesions are most marked in the lungs and kidneys and may even be confined to them. Very large doses of human bacilli (10 to 50 mg. intraperitoneally) may produce a progressive infection but not the acute fatal miliary disease. The mammalian tubercle bacilli, indistinguishable by cultural or serological methods, may, then, be

Characteristics of the Varieties of Tubercle Bacilli

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VARIETY	OPT. TEMP.	RATE OF PIG- GROWTH MENT	STIMULA- TION BY GLYCEROL	GUINEA RABBIT CHICKEN PIG	
Human Bovine Avian	37°C. 37°C. 40-42°C.	Eugonic + Dysgonic - Rapid +	<u>+</u>	7+1++ # # # # # # # # # # # # # # # # # #	

sharply differentiated by their pathogenicity for these two experimental animals.

Neither rabbit nor guinea pig is particularly susceptible to infection with the avian bacillus, though avian tuberculosis may be produced in the rabbit.⁴² In the guinea pig death may be produced by large intraperitoneal doses, but on autopsy macroscopic tubercles are not visible; cultures and smears of the liver and spleen, however, show the presence of the bacilli. This form of tuberculosis, the proliferation of the bacilli without macroscopic tubercle formation, is known as the Yersin type of tuberculosis.

Experimental infections of mice inoculated with the human variety of tubercle bacilli have been studied at length by Youmans and his co-workers. 167, 168 They have found that the median survival time of the infected animals is a linear function of the logarithm of the dose and that the generation time of the tubercle bacillus is four to six days *in vivo* in this animal as compared to 14 hours *in vitro*. These precise studies have made the mouse a valuable experimental animal, and it has been used extensively in work on chemotherapeutic drugs.

Epidemiology. 44, 55, 160 In man tuberculosis is largely an airborne infection. Dissemination by milk is now of relatively minor importance in the United States. The disease is one of civilization in that its transmission is facilitated by close contact. The frequency of positive tuberculin reactions rises rapidly from zero in the newborn through puberty, and many adults have been infected at one time or another. In most instances clinical tuberculosis has not occurred and the lesions have healed. The proportion of persons with active clinical tuberculosis is not known; in some investigations the ratio of cases to deaths has been found to be as high as 10:1 or 12:1, but the ratio of reported cases to deaths is not much more than half of this. The prevalence of tuberculosis, then, defies precise definition. Active tuberculosis, as found at autopsy, is also appreciable; in 1960 4:4 per cent of the cases in New York, and in 1963 10 per cent of the cases in Baltimore, were reported only after death. 129

There are marked racial differences in the prevalence of tuberculosis. The death rate of Negroes in this country is considerably higher than that of the white population, though the incidence of clinical tuberculosis in the two races is not greatly different. Whether this higher death rate is a consequence of environmental conditions or

whether it is in part attributable to racial differences in susceptibility has been a point of considerable interest. In this connection the experience in the United States Army is of particular interest. In the years 1922 to 1936 the average white morbidity rate was 2.10 per 1000 and the Negro rate 2.56, a ratio of 4:5; the white death rate was 0.24 and that of the Negroes 0.99, a ratio of 1:4; and the case-death ratios were 8.75 for the whites and 2.61 for the Negroes. Under the controlled conditions prevailing - i.e., preliminary physical examination, age, and sex selection, the same housing conditions, and identical diagnostic and therapeutic facilities -it would appear that though the incidence of clinical tuberculosis is not greater in the Negro, the disease is more frequently fatal. and the indicated greater susceptibility is a consequence of racial rather than environmental factors. During World War II the Negro, while making up but 10 per cent of the United States Army, contributed 43.4 per cent of the total deaths from tuberculosis. There is some evidence that less well-defined races of man differ in their resistance to tuberculosis: Jews and Italians appear to be more resistant than the Irish.

Tuberculosis has been decreasing at a steady and relatively rapid rate since 1850 or thereabouts as indicated by the decline in the death rate from this disease.^{38, 65} As in the case of some other diseases, the decline set in before the discovery of the bacterial etiology of infectious disease and the development of preventive measures, and hence is by no means entirely attributable to the practice of preventive medicine.

This decline has not been relatively the same in either the various age groups or in the two sexes. The death rate is highest in the very low age groups, that of one to two years, and falls rapidly in the five to nine group and then rises to a peak in early adult life, 20 to 24, and declines with, in recent years, a small secondary peak between 45 and 54 years (late adult tuberculosis). The decline in tuberculosis in the present century has been relatively greatest in the very young, an undoubted consequence of preventive measures.⁴⁰

The sex distribution of the death rates from tuberculosis in the various age groups is a curious phenomenon which has not been explained. The death rate for males of all ages is somewhat higher than that for females. In young adults, i.e., the 15 to 29 age group, the female death rate is consid-

erably higher than the male death rate. In the higher age groups the male death rate becomes proportionately greater and exceeds the female rate for the rest of life. In the present century the female death rate in the higher age groups has declined somewhat more rapidly than the male death rate.

There is no simple explanation for the observed decline in tuberculosis mortality. Probably the isolation of active cases to reduce the source of infection, together with improvement in living conditions, both environmental and nutritional, has played a significant part. Such a decline is usually taken as a manifestation of the inability of a disease to reproduce itself and leads to the prediction of its eventual disappearance. This need not necessarily follow, however, and in this country the number of new cases reported has increased since 1940. For example, between 1940 and 1947 the number of deaths declined by 20 per cent, while the number of new cases reported rose from 100,772 in 1940 to 133,837 in 1947 and then declined to 55,494 in 1960.

In this country, England, and the Scandinavian countries there appears to be a reservoir of minimal undiagnosed active tuberculosis of the late adult type concentrated in the age groups over 45 that is increasing.86, 107, 112 In New York City, for example, the proportion of new cases and deaths in persons over 45 doubled in the 20-year period prior to 1950, and 65 per cent of all deaths were of persons over 45. of whom 85 per cent were male. If this represents a residual of higher rates in earlier life, it will eventually decline, but if it is a consequence of suppression of the disease in otherwise susceptible persons by environmental factors, the reservoir of such persons may increase. It represents a source of infection to a population becoming increasingly more susceptible as a consequence of reduction in immunizing infections.

MYCOBACTERIUM LEPRAE (Hansen's Bacillus)^{26, 61, 68}

Like tuberculosis, leprosy is an old disease of man. The first accurate description of the disease in India was in the Sushruta Samhita about 600 B.C.; 130 it was known in Egypt in the time of the Pharaohs, and in China in the fifth century B.C. Perhaps more prevalent in ancient times, it is now most common in

Central Africa, India, Japan and other Asiatic countries, and the South Pacific. The disease is prevalent in South America, with endemic centers in Brazil, Colombia, and Argentina. It is estimated⁸ that there are 80,000 cases in Brazil, 50,000 in Mexico, 16,000 in Argentina, 12,000 in Colombia, 10,000 in Paraguay, 6000 in Cuba, and 3400 in Peru. It is relatively uncommon in Europe, and sporadic cases occur in Latvia, Estonia, and southern and eastern Russia, and on the Mediterranean coast. The total number of lepers in the world is not accurately known; estimates vary from two to seven million.

Leprosy has been introduced into the United States with varying consequences. In Louisiana, Florida, and Texas the imported cases have established foci in which the disease has a tendency to perpetuate itself, while in California and the central northwestern states it tends to die out. Elsewhere in the country transmission is so rare as to be negligible. It is estimated that there are 500 to 1000 lepers in this country, many of whom are segregated in the National Leprosarium in Carville, Louisiana, the remainder living for the most part in California and New York.

Bacilli were found by Hansen in 1872 in the round epithelioid cells generally known as lepra cells, and the observation was one of the first of pathogenic bacteria.

and staining. 132 Morphology Morphologically the leprosy bacilli closely resemble the tubercle bacilli. They are long (6μ) , slender rods, usually straight, but sometimes slightly curved, and club-shaped forms are found occasionally. 159 They are nonmotile and do not produce spores. They generally occur within the cells but are sometimes found free in the lymph spaces. Their arrangement within the cells is characteristic. several bacilli being usually grouped together in bundles like packets of cigarettes. Capsules are present on cells which are presumed to be viable, but are destroyed by the use of hot carbol-fuchsin for staining.53

The staining reaction of these microorganisms is much like that of the tubercle bacilli. They stain somewhat more readily than the latter, and also decolorize more quickly with acids, but the difference is not great enough for differentiation. The presence of large numbers of bacilli within the cells, together with the clinical features of the disease, makes it possible to distinguish leprosy bacilli from tubercle bacilli without difficulty.

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Because of their acid-fast staining characteristic and their morphological resemblance to the tubercle bacilli and similar bacteria, these bacilli are included with the mycobacteria designated *Mycobacterium leprae*.

Cultivation. Numerous unsuccessful attempts to cultivate Hansen's bacillus on artificial mediums were made for years by bacteriologists all over the world. A few investigators have reported positive results. In most instances acid-fast bacilli have been cultivated, but in others a variety of microorganisms, including diphtheroids, actinomycetes, and anaerobic bacilli, have been found. It has been suggested by some workers that the acid-fast bacilli present in leprous lesions represent but a single stage in a developmental cycle and that outside the body other forms appear. There are at present a number of cultures in various laboratories labeled Myco. leprae. It seems highly probable that none of these bacteria are leprosy bacilli in that they are etiologically related to the disease in man. They are very likely best grouped with the saprophytic acid-fast forms such as the smegma and timothy bacilli.

Among the more recent studies on the isolation of leprosy bacilli are those of de Souza-Araujo, 134 who has isolated acid-fast bacilli that have produced suggestive reactions on inoculation into monkeys. Freire 49 has cultivated similar bacilli in slide cultures. There have been a number of reports of cultivation, *i.e.*, persistence with apparently a few cell divisions, in tissue culture. 5, 37, 101

Pathogenicity. Although any organ or tissue in man may be attacked with varying results, two distinct types of leprosy are usually recognized—the nodular and the anesthetic. The former, which is the more acute, is characterized by the development of masses of granulation tissue, the so-called leproma, which may appear superficially in different parts of the body and by their growth and coalescence produce distortion and mutilation. The anesthetic type, or nerve leprosy, progresses more slowly, the average duration of the cases being nearly twice as long (18 years) as cases of the nodular type, some being known to extend over 35 or 40 years. Atrophy of the muscles and other trophic disturbances accompany the nerve

In both forms of leprosy Hansen's bacillus is found in all cases, in enormous numbers as a rule in the lesions of nodular leprosy and less abundantly in the anesthetic type. Very

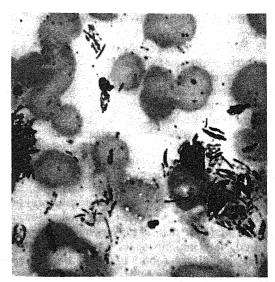


Figure 182. The leprosy bacillus. Acid-fast stained smear from a skin lesion. Note the characteristic tendency to parallel arrangement of the bacilli in packets. \times 1800.

few bacilli are observed outside the body cells, and they are found in the cytoplasm and do not invade the nucleus. Almost any part of the body may be the site of leprous growth; the kidneys are usually invaded, the liver and spleen always. The bacilli have been seen in the central nervous system and are occasionally found in the blood, generally in the leucocytes but sometimes free. There are a number of classifications of the clinical disease. 14, 21, 71, 110

Experimental infections. Until relatively recently it has not been possible to produce lesions with multiplication of leprosy bacilli in experimental animals by inoculation of leprous material. It has been found that inoculation of the footpads of mice results in an increase in numbers of the bacilli over relatively long periods of time. 123 The infection is transmissible and is inhibited by prior im-

Relative Proportion of Types of Leprosy*

	NEURAL	LEPROMATOUS		
REGION	TYPE %	TYPE %		
Africa	90.5	9.5		
Philippines	50.0	50.0		
Mexico	40.0	60.0		
Java	29.0	71.0		

^{*}Data from Lowe: Proc. Sixth Pacific Sci: Congr., 1942, 5:921.

munization with BCG126 or with antituberculosis drugs. 127 This experimental infection in mice has been confirmed by some, 96 but not by others. 67, 68 Similar infections have been produced in hamsters. 32, 153, 154 in the ears as well as footpads, and there is evidence that the optimum temperature for growth of the bacilli is less than body temperature. 125 It has been suggested that past failure to produce experimental infection by the inoculation of human leprous material was attributable not only to unfavorable temperatures in the deeper tissues, but also to the use of material from the tuberculoid or lepromatous types of the human disease. Such material may largely contain fully matured, terminal forms of the bacilli rather than bacilli, as found in borderline cases, more readily adaptable to another host.31

Transmission. Leprosy is probably transmitted by contact, though the conditions that make transmission possible are entirely unknown. There are numerous instances in which healthy persons, such as asylum attendants, have been more or less in contact with lepers for long periods without contracting the disease. In other cases, however, leprosy has been contracted by those in close and long-continued contact with diseased individuals. There is also indirect evidence of transmission. Manson cites the case of an Irishman who acquired leprosy in the West Indies. On his return to Ireland his bed was shared by his brother who, moreover, sometimes wore the leper's clothes. The brother. who had never been in any foreign country. became in time an undoubted leper. In this instance communication from one person to another was practically demonstrated.

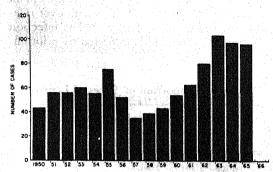


Figure 183. Cases of leprosy reported annually in the United States in the period 1950-1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U. S. Department of Health, Education, and Welfare.)

A large percentage of cases studied in Hawaii gave a history of exposure, and usually such exposure was of an intimate character. Leprosy occurs for the most part in populations of low social order living under unsanitary conditions of poverty, crowding. uncleanliness, and sickness. Even so, a relatively small proportion of those exposed develop the disease, and conjugal leprosy is unusual.85 Age at the time of exposure is important, the risk being greatest for those exposed before five years of age. Of children of lepers who are separated from their parents and who develop leprosy subsequently. the disease appears within the first year in 50 per cent, and in 90 per cent within three vears, after the last exposure.97

The bacilli may often be found in nasal secretions in analogy to tuberculous sputum. Although there has been some tendency to discredit nasal mucus as a source of infection, more recent studies indicate the importance of such secretions, the numbers of leprosy bacilli excreted per day approximating those of tubercle bacilli in sputum. 124 Whether the nasal area represents a primary site of infection is another matter. 16 For diagnostic purposes not only nasal smears. but scraped incision skin smear^{51, 66, 95} from a number of sites, as carried out at Carville, is preferable, and may allow the diagnosis of grossly inapparent infections. 60

Evidence of the direct inoculability of

leprosy from man to man is quite inadequate. Many attempts to infect healthy persons have been made and have failed, and one often cited instance of successful inoculation is by no means unimpeachable. In the case of the criminal Keanu in the Hawaiian Islands, reported by Arning in 1893, implantation of material from a leprosy nodule was followed by the development of true leprosy. which terminated fatally six years after inoculation. The experiment, however, did not include the important source of error involved in the facts that Keanu was a native of a country in which leprosy was common. that he had lived among lepers, and that members of his family were lepers. Later Lagoudaky⁷⁰ inoculated himself intramuscularly with blood from two lepers and contracted the disease.

Much light is thrown on the contagious character of leprosy by the success that has attended the isolation and segregation of leprous patients. The Norwegian experience showed that a careful but not unduly rigorous system of separation was accompanied by

a diminution of the number of cases from 2870 in 1856 to 577 in 1900 and to seven in 1964. 149 The circumstance that infection does not invariably follow chance contact or association should not, therefore, lead to the neglect of these facts: that leprosy is a bacterial disease; that up to the present under natural conditions the specific bacterium has not been found except in the human body; and that, so far as is definitely known, the leper himself is the most important means by which leprosy spreads.

Chemotherapy.^{24, 39} The similarities between the acid-fast bacilli observed in leprosy and the tubercle bacillus inevitably suggested the use of the drugs effective in tuberculosis in the chemotherapy of leprosy. Of these, the sulfones have been the most satisfactory, usually giving relatively rapid clinical improvement and slower bacteriological improvement. There is some tendency to relapse following cessation of chemotherapy. Diaminodiphenyl sulfone (DDS, Dapsone) is considered to be the chemotherapeutic agent of choice, and the activity of various derivatives seems to be attributable to the extent to which degradation to the parent compound occurs in vivo.

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The thiosemicarbazones appear to be reasonably effective but less so than the sulfones and are regarded as useful as an alternative when sulfones are not tolerated. Para-aminosalicylic acid has little chemotherapeutic activity, and preliminary studies with isonicotinic acid hydrazide have not been encouraging. Streptomycin has appreciable chemotherapeutic activity but has been found too toxic for the long period of administration required.

Immunity.²⁵ Man is highly resistant to infection with the leprosy bacillus, and, after infection is established, the ensuing disease is essentially a benign process that may take many years to terminate fatally; in fact, many lepers die from other causes. Little is known regarding the specific immune response. Lepels do, however, develop a hypersensitivity to the cell substance of acidfast bacilli such as the tubercle bacillus and the various saprophytic species. Muir⁸⁷ has suggested that the acid-fast bacilli exist as a continuous series with respect to parasitism, ranging from the saprophytic forms through the chromogenic forms occasionally found associated with the disease, the tubercle bacilli of clear host specificity, to

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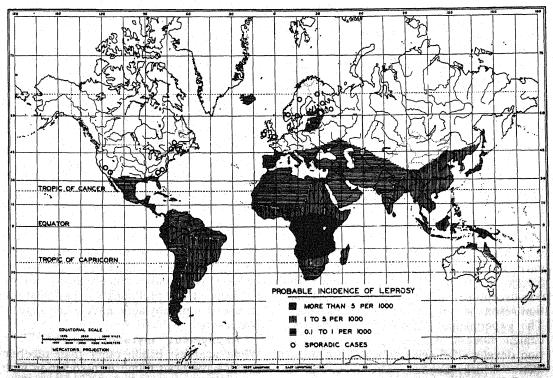


Figure 184. The probable prevalence of leprosy in the world. (Based on Goode Base Map No. 201 M. By permission of the University of Chicago Press.) (After Saunders.)

the leprosy bacillus which appears to be unable to live outside the host tissue. It is perhaps a consequence of the immunological relationships among the acid-fast bacilli that there appears to be a mutual exclusion between leprosy and tuberculosis. 15, 52 A curious fact is the appearance of a positive Wassermann reaction in lepers in the absence of syphilis, and both the Wassermann and the Kahn tests may be positive in lepers having no clinical symptoms of syphilis; moreover the serum of lepers is often anticomplementary.

Lepromin. 142, 143 The hypersensitivity developed by the leper may be demonstrated by the intradermal inoculation of material prepared from leprous nodules. Two reactions are observed, one early and occurring after three or four days, and the late reaction appearing three to four weeks after inoculation. The first is the Fernandez reaction and the second the Mitsuda reaction. The Mitsuda reaction is regarded by some workers as an expression of immunity, but it is nonspecific in that it occurs in tuberculosis and is induced by immunization with BCG.

MYCOBACTERIUM LEPRAE MURIUM

A native disease of wild rats, commonly known as rat leprosy, and characterized by enormous numbers of acid-fast bacilli present in the lesions, was described by Stefansky in 1903 as occurring in wild rats in Odessa. The disease was observed in the same year by Dean, who later showed it to be transmissible. It has since been observed in wild rats all over the world.⁴⁵

The acid-fast bacilli closely resemble the leprosy bacillus in size and shape and are found intracellularly but not as often in the packet arrangment of parallel bacilli. 19, 133 Myco. leprae murium has not been cultivated on artificial mediums64 but has been reported50, 54, 150 to persist through a few cell divisions in tissue culture. The disease may, however, be transmitted to white rats, mice, and guinea pigs by inoculation with pieces of tissue. Wild mice are relatively nonsusceptible, and subcutaneous inoculation results in only a transient granuloma.92 The experimental infection is a relatively benign process. A local, circumscribed lesion develops following subcutaneous inoculation which becomes palpable in four to five weeks and eventually develops to a large tumorous mass ulcerating on the surface and persisting throughout the life of the rat. The

earliest lesions in the other organs do not appear before four to six months, and the animal dies only after a year or more.¹⁸

The relation of rat leprosy to human leprosy is not known. Rats are not susceptible to inoculation with human leprous material.

OTHER ACID-FAST BACILLI

Mycobacterium paratuberculosis. A chronic enteritis of cattle usually terminating fatally is caused by an acid-fast bacillus closely resembling the avian variety of the tubercle bacillus. The disease is sometimes called Johne's disease and the organism Johne's bacillus after its discoverer. The disease only remotely resembles tuberculous infection. The lesions in the intestinal wall are proliferative, and the granulomatous tissue may contain epithelioid cells and occasionally giant cells, but there is no caseation.

The disease appears to be widespread in the United States. Infected cattle become hypersensitive to the bacillary substance, and filtrates of cultures produce a skin reaction analogous to the tuberculin reaction which is designated the "johnin reaction." No case of human infection with Myco. paratuberculosis has been recorded.

The vole bacillus. An acid-fast bacillus responsible for an epizootic, chronic infection of the field vole, Microtus agrestis, resembling tuberculosis was discovered by Wells in 1937. It closely resembles the tubercle bacillus culturally though it forms no pigment and growth is not enhanced by glycerol. It is pathogenic for both guinea pigs and rabbits, considerably more so for the latter, and is not pathogenic for fowls. It has been suggested that it is a distinct type of mammalian tubercle bacillus and should be called Myco. tuberculosis var. muris. This microorganism has been of particular interest because, though it produces only a localized and retrogressive infection when inoculated in small doses in guinea pigs and calves, tuberculin sensitivity is produced, and preliminary experiments on its use as a prophylactic have given suggestive results.

"Anonymous" mycobacteria. 20, 56, 63, 165
While acid-fast bacilli other than tubercle
bacilli have been found in association with
human disease from time to time, they have
been isolated more and more often within
the past few years and have attracted in-

creased attention. Because they are not identifiable with known species, they have been known as the "anonymous," "atypical," or "unclassified" mycobacteria.

These bacteria are found in man in the absence of disease, admixed with tubercle bacilli in tuberculous infections, and in large numbers in pure culture in apparently etiologic relation to tuberculous-like disease. The pulmonary disease^{33, 131} closely simulates tuberculosis, but endobronchitis seems to be more common, and there is somewhat more nonspecific inflammation and fibrosis. Similarly, the pathology of lymphatic infection, resembles that of tuberculous infection, including calcification in the later stages, but the infected nodes tend more often to be necrotic and suppurative in character.81 These infections are not common, and are reported to make up 1 per cent or less of suspected tuberculosis.

A granulomatous type of disease called "swimming pool granuloma" is well recognized and is attributed to the "cold-blooded" form *Myco. balnei*. The relationship of this microorganism to *Myco. fortuitum* of the atypical group IV (see below) is uncertain, ^{128, 141} as is that of *Myco. ulcerans*, the causative agent of necrotizing skin ulcer known as Buruli ulceration, found in Africa. ^{30, 98} Similarly, there are close resemblances between the "Battey" bacillus of group III and the avian tubercle bacillus. ^{13, 115}

These bacteria differ from the human tubercle bacilli in that they are not pathogenic for the guinea pig, are relatively resistant to the antituberculosis chemotherapeutic agents, grow more rapidly, and some are pigmented.^{28, 77} They are separated by Runyon into four groups, *viz.*:

Chromogens:

Group I. Photochromogens, producing a bright yellow pigment in the presence of light; many strains are pathogenic for man and the mouse; includes Myco. kansasii (Myco. luciflavum).

Group II. Skotochromogens (scotochromogens), producing a reddish-orange pigment independent of light; usually nonpathogenic for man or the mouse; includes *Myco. scrofulaceum*.

Nonchromogens:

Group III. Filamentous forms, possibly including some Nocardia strains; some strains pathogenic for man and the mouse; includes the Battey bacillus and Myco. (Nocardia) intracellularis.

Group IV. The "rapid growers," characterized by very rapid (2-4 days) growth; a few strains patho-

genic for man and the mouse; includes Myco. fortuitum.

These bacteria are immunologically related to the tubercle bacilli and other mycobacteria, 6, 166 and are considered by some 161 to have arisen from the former.

"Cold-blooded" mycobacteria. Acid-fast bacilli have been found associated with pathological processes in various cold-blooded animals. In some instances the processes superficially resemble tuberculous lesions. Myco. piscium was isolated from nodules and tumor-like formations in carp; Myco. marinum from "tuberculosis" of sea bass and certain other salt-water fish; Myco. ranae was found in the liver of a frog; Myco. thamnopheos is a parasite in garter snakes; and Myco. chelonei is the so-called turtle bacillus.

Saprophytic acid-fast bacilli. Included in this category are the well known timothy bacillus, Myco. phlei, found in soil, on grasses, and elsewhere in nature; the "butter bacillus," Myco. butyricum; and Myco. smegmatis, which is, however, a parasite found in both male and female smegma. The smegma bacillus is often difficult to distinguish from the tubercle bacillus on morphological grounds, and confusion of the two may have considerable practical importance in the diagnosis of suspected cases of tuberculous infection of the urinary tract. It is, it may be noted, also found in the urine and may contaminate fecal specimens. The saprophytic bacilli all grow much more rapidly than the tubercle bacilli, and neither they nor the bacilli isolated from coldblooded animals are pathogenic for guinea pigs and rabbits, or at best only feebly so.

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Chapter Thirty-two

MEDICAL MYCOLOGY: THE PATHOGENIC FUNGI AND THE PATHOGENIC ACTINOMYCETES

By JOHN W. RIPPON, Ph.D.

The discovery of the causal relation of certain of the fungi to infectious disease preceded the pioneer work of Pasteur and Koch with the pathogenic bacteria by several years, for Schoenlein and Gruby studied the fungus causing favus (Trichophyton schoenleinii) in 1839, and in the same year Lagenbeck described the yeast-like microorganism of thrush (Candida albicans). Prior to this, Bassi described muscardine of silkworm (caused by Bauvaria bassiana). In spite of its earlier beginnings, medical mycology was soon overshadowed by bacteriology and has never received as much attention, though some of the fungous diseases are among the more common infections of man. This is perhaps attributable to the relatively benign nature of the common mycoses and the rarity of the more serious ones, and to the morphological basis of the differentiation of these structurally complex forms which, in a practical sense, sets them off sharply from the bacteria.

Even a brief consideration of the fungous diseases makes it clear that they fall into two well-defined groups, the superficial mycoses and the deep-seated mycoses. The superficial mycoses are by far the more common; are caused for the most part by a relatively homogeneous group of fungi, the dermatophytes; and include the various forms of tinea or ringworm, with infection of the hair and hair follicles, the superficial infections of the intertriginous or flat areas of the glabrous skin, and the onychomycoses

or fungous infections of the toe- and fingernails. In general, the lesions are mild, superficial, and restricted: the infections are almost never fatal, although invasion of the brain and heart by Trichophyton violaceum has been reported from Russia, Japan, and Roumania.8 The causative microorganisms are specialized saprophytes with the peculiar ability to digest keratin and which ultimately have their reservoir in soil; however, infections are frequently transmitted from one host to another. A yeast, Candida, also produces a dermatophyte-like disease. The deep-seated mycoses, on the other hand, are of sporadic distribution, being very common in some parts of the world and unknown in other areas, and are of heterogeneous etiology. They include histoplasmosis, aspergillosis, sporotrichosis, blastomycosis, coccidioidomycosis, cryptococcosis, and candidiasis (systemic) infections. The causative microorganisms appear to be soil-inhabiting saprophytes with peculiar abilities for adaptation to the internal environment of their host and are usually not transmitted from one individual to another.2 Infection by the organism in an endemic area may be very common however. Few infections develop into the severe, deep, spreading, and often fatal disease seen in some individuals.

A third group of microorganisms also treated in this chapter are the actinomycetes. These are bacteria which cause fungus-like infections and which were originally con-

sidered intermediate organisms. Morphologically, physiologically, and biochemically they are bacteria and are susceptible to antibacterial antibiotics; fungi are not.

Two of the diseases discussed in this chapter, actinomycosis and candidiasis, are caused by endogenous organisms, that is, species which are part of the normal flora of man; all other fungous and actinomycetous infections are exogenous in origin.

The criterion of pathogenicity is one of the poorest that can be used in the differentiation of microorganisms, not only because it is variable and difficult to determine, but because by its use parasitic microorganisms are grouped together that are, in fact, much more closely related to certain freeliving forms than they are to one another. The superficial nature of pathogenicity as a differential characteristic is nowhere better illustrated than among the fungi, for the pathogenic forms which constitute the subject matter of medical mycology form a heterogeneous group that includes some of the actinomycetes, certain molds and moldlike fungi, and a number of yeasts and yeastlike organisms. Of the nearly one hundred thousand species of fungi only a very few are known to be frequent infectious agents. of man and higher animals. With the possible exception of two or three dermatophytes, none of this group is an obligate parasite and most are misplaced soil saprophytes. The dermatophytes are frequently contagious, and man may serve as a disseminator of the species. Infection by the agents of the deep mycoses, however, seems to be accidental and is a blind alley, the parasite dying with its host. From a general biological point of view, then, the pathogenicity of certain fungi is of very minor significance; from that of the parasitized host, man, it is of considerably greater interest.⁷

The fungi are structurally complex, showing a variety of reproductive structures associated with sexual and asexual processes in addition to vegetative nonreproductive elements. Their differentiation into genera, species, and varieties is made in large part on a morphological basis,^{3, 4, 6} especially the morphology of the reproductive structures and, in contrast to the bacteria, their physiological and immunological characteristics are usually of minor or no importance for purposes of differentiation or identification. The biochemistry of the fungi, and of the molds in particular, has been extensively investigated in connection

Clinical Types of Fungous Infections

TYPE	DISEASE	CAUSATIVE ORGANISM	
Tinea versicolor Superficial infections Piedra		Pityrosporum orbiculare (Malassezia furfur) Trichosporon cutaneum (white) Piedraia hortai (black)	
Cutaneous infections	Ringworm of scalp, glabrous skin, nails	Dermatophytes. Microsporum, Trichophyton, Epidermophyton	
	Candidiasis of skin, mucous membranes, nails; sometimes generalized	Candida albicans and related forms	
Subcutaneous infections	Chromoblastomycosis	Fonsecaea pedrosoi and related forms	
	Mycotic maduromycosis	Allescheria boydii, Madurella myce tomi, et al.	
	Subcutaneous phycomycosis	Basidiobolus meristosporus	
Deep-seated	Histoplasmosis	Histoplasma capsulatum	
infections	Blastomycosis, North American	Blastomyces dermatidis	
	Blastomycosis, South American	Paracoccidioides brasiliensis	
	Coccidioidomycosis	Coccidioides immitis	
	European blastomycosis	Cryptococcus neoformans	
	Sporotrichosis	Sporotrichum schenckii	
	Aspergillosis Phycomycosis	Aspergillus fumagatus, etc. Mucor spp., Absidia spp., Phicopus spp.	

with the elucidation of decompositions occurring in nature and their application to industrial processes. Present knowledge of the respiratory mechanism of the cell and of alcoholic fermentation derives in very large part from studies of yeast. The immunological properties of the fungi have been little studied aside from noting the allergic phenomena associated with infection by certain of the dermatophytes and yeast-like fungi and frank allergy, i.e., asthma associated with fungal spores.

The skin test and complement fixation test, which are occasionally helpful in diagnosis, have been the subject of much investigation. Fungi are generally not good antigens and the role of immune mechanisms in

fungous disease is unclear. 133

The tissue response of the host to the offending agent varies widely with the variety of organism. In dermatophyte infections, erythema is generally produced and is probably a response to the irritation of the organisms or its products of metabolism. Occasionally, severe inflammation, followed by scar tissue and keloid formation, will occur. In organisms that invade living tissue, such as those responsible for subcutaneous and systemic disease, there is generally elicited a rather uniform acute pyogenic response. This usually gives way to a variety of chronic disease responses, listed in the accompanying outline. 19

Tissue Reactions in Fungous Diseases*

Chronic inflammation

Lymphocytes, plasma cells, neutrophils, and fibroblasts; occasionally giant cells

Rhinosporidium seeberi Subcutaneous phycomycosis

Pyogenic reaction

Acute or chronic, suppurative neutrophilic infiltrate

Actinomyces israeli sulfur granules
Nocardia astroides
Acute aspergillosis
Acute candidiasis

Mixed pyogenic and granulomatous reaction

Neutrophilic infiltration and granulomatous
reaction, lymphocytes, plasma cells

Blastomyces dermatitidis Paracoccidiodes brasiliensis Coccidioides immitis: neutrophils, especially at broken spherule

Sporotrichum schenckii: organism rarely seen in tissue

Chromoblastomycosis: chronic pyogenic, inflammation, epithelioid cell nodules and giant cells

Maduromycosis; in addition may be large foamy giant cells similar to xanthoma

Pseudoepitheliomatous hyperplasia

Following chronic inflammation in the skin hyperplasia of epidermal cells, hyperkeratosis, extension of rete pegs

B. dermatitidis
P. brasiliensis
Chromoblastomycosis
C. immitis

Histiocytic granuloma

Histiocytes frequently, with intracellular organisms sometimes becoming multinucleate giant cells

Histoplasma capsulatum Meningeal C. neoformans

Granuloma with caseation

Granulomatous reaction, Langhans' giant cells (L.G.C.), central necrosis

Histoplasma capsulatum Coccidioides immitis

Granuloma "sarcoid" type
Non-necrotizing

Cryptococcus neoformans Occasionally histoplasma

Fibrocaseous pulmonary granuloma; "tuberculoma"

H. capsulatum: thick fibrous wall surrounding epithelioid and L.G.C. organisms in soft center, often calcification

C. immitis: thin fibrous wall, rarely calcified

C. neoformans: poorly defined

Thrombotic arteritis

Thrombosis, purulent coagulative necrosis, invasion of vessel

Aspergillosis Phycomycosis

Fibrosis

Proliferating fibroblasts, deposition of collagen -- may resemble keloid

Loboa loboi

Sclerosing foreign body granuloma
In paranasal sinuses or viral infection

Aspergillus sp., bizarre hyphae in giant cells

Though a great many species of fungi have been described as pathogenic for man and animals, not all are of equal importance.

^{*}A Gram stain is used for actinomycosis, nocardiosis, actinomycotic mycetoma, and candidiasis; otherwise a PAS stain is recommended.

Some, especially among the dermatophytes, are not legitimate species different from those already known. Furthermore, in many cases the fungus described probably had no causal relation to the pathological process from which it was isolated, and in others only one or two cases of infection have ever been observed. It is sometimes very difficult to decide whether a fungus isolated from clinical material is of any importance. The accumulation of large numbers of species

of fungi and the minutiae of their morphological differentiation give medical mycology its complexity. Many skin, sputum, and air contaminants have been written into the literature as disease-producing organisms. Here we shall be concerned only with the more important fungi known to be causally related to human disease⁷; the remainder, though large in number, are associated with only a small fraction of the fungous diseases of man.

The Pathogenic Actinomycetes

The human pathogenic actinomycetes are so-called "higher" bacteria and are classified in the order Actinomycetales. This order includes some chronic disease-producing organisms such as the etiological agents of tuberculosis and Hansen's disease (leprosy). By tradition these last two organisms have been studied along with other bacteria. The remaining pathogenic actinomycetes, however, were thought to be transitional forms between bacteria and fungi and were included in the sphere of medical mycology. The etiological agents of lumpy jaw and actinomycotic mycetoma show some funguslike characteristics such as the branching of the organism in tissue, the extensive mycelial network that may occur in tissue or in culture, and the chronicity of the disease produced. However, cell wall analysis shows the presence of the typically bacterial muramic acid, which, along with the lack of a structural nucleus, typical bacterial size, and susceptibility to antibacterial antibiotics, defines these organisms as bacteria and not fungi. As far as their role as a phylogenetic "link" to the fungi is concerned, the presence of typical eucaryotic nuclei and mitochondria in fungous cells makes the derivation of the fungi from procaryotic bacteria independent of other eucaryotic organisms extremely improbable.

Morphology. The organisms grow in the form of fine straight or wavy nonseptate filaments or hyphae 0.5 to $0.8~\mu$ in diameter which show both lateral and dichotomous branching and which may grow out from the medium to form an aerial mycelium. On solid mediums the filaments occur in tangled masses, while in liquid mediums there is a tendency to centers or clumps of growth. There are three genera of medical interest: the anaerobic Actinomyces and the aerobic

Nocardia and Streptomyces.²³⁰ Classification of species within the genera and even separation of the genera themselves is controversial.²³¹ The following characteristics are generally accepted by workers in the field. Actinomyces includes organisms which are anaerobic or micro-aerophilic and nonacid-fast and in which the vegetative mycelium breaks up into bacillary or coccoid elements. The Nocardia are aerobic, sometimes partially acid-fast, and they fragment into bacillary or coccoid forms and produce chains of squared spores 1 to 2 μ long by simple fragmentation of hyphal branches.14 In Streptomyces there is more aerial mycelium, no fragmenting to bacillary or coccoid forms, and no acid-fastness, and chains of round to oval spores are produced consecutively within a specialized hyphal element. The maturation of the spore-bearing hyphae is often associated with the formation of spirals which range from open, barely perceptible coils to those which are so compressed that adjacent turns are in contact. The spirals may be dextrorse or sinistrorse; and both the direction and the tightness of coiling are constant within species. The spores are more resistant than the filaments and will survive 60° C. for as long as three hours, but are less resistant than bacterial spores. The tips of other filaments may become swollen and club-shaped. Segmentation of the filaments occurs in some species as early as 24 hours, while in others it is delayed three weeks or more; the segmented filaments fragment to form bacillary bodies 4 to 6 μ in length which are morphologically indistinguishable from many other bacteria. In most smear preparations of the pathogenic forms, the filaments are broken up and the appearance is that of ordinary bacilli. All, or practically all, of the actinomycetes are gram-positive, and some of the pathogenic forms are partially acid-fast.

Both spores and fragments of mycelium grow in subculture. On solid mediums the growth of the aerobic forms is dry, tough, and leathery, sometimes wrinkled, adherent to and piled above the medium; in many instances it resembles the growth of mycobacteria. In some cases the growth appears powdery or chalky, owing to the formation of aerial mycelium. In liquid mediums growth occurs in the form of a dry, wrinkled surface film or, more often, as flakes or aggregates which adhere to the sides of the flask, especially at the surface, or sink to the bottom.

Pigment formation, with colors ranging over the entire spectrum, is common among the actinomycetes, and differentiation is usually made between pigmentation of the vegetative mycelium and the spore-bearing aerial mycelium, as well as on pigment diffusing into the medium. Soluble purple and brown pigments are often observed on protein-containing mediums. The actinomycetes, especially the saprophytic forms, are physiologically active, utilizing a variety of carbon and nitrogen compounds, and many are actively proteolytic. An earthy to musty odor, like that of freshly turned soil or of a damp basement, is produced by many species. The optimum temperature for growth is usually 20° to 30° C. though some of the pathogenic species grow at 37° C., and thermophilic species, analogous to thermophilic bacteria, are known. The great majority of actinomycetes are aerobic, but some of the pathogenic forms are anaerobic or at least must be cultivated under reduced oxygen tension.

Differentiation of the actinomycetes from one another is determined in part on a morphological and in part on a physiological basis, the latter including pigmentation and proteolytic activity.

ACTINOMYCOSIS171

Lumpy jaw or actinomycosis was once a fairly common disease of cattle and occasionally of man. Today it is an uncommon infection, most often diagnosed in retrospect. This change is largely due to the widespread practice of giving antibiotics indiscriminately. The etiological agents Actinomyces israeli, A. bovis, and A. eriksonii are quite sensitive to most antibacterial antibiotics including penicillin and the sulfas. Formerly,

infection was often associated with tooth extraction or dental surgery, which provided traumatized tissue in which these endogenous organisms could grow. Prophylactic antibiotics following oral procedures have eliminated this hazard. Although the disease was undoubtedly observed early in the nineteenth century, actinomycotic tumors being described by Leblanc in 1826 under the name of osteosarcoma, it was first recognized as a specific parasitic disease by Bollinger in 1877. At his instigation the organism was studied by the botanist Harz, who described it and named it Actinomyces or ray fungus, because of the ray-like structure of its growth in the tissues, but he did not cultivate it. In 1891 Wolff and Israel isolated a micro-aerophilic actinomycete from pathological material by anaerobic culture, and in the same year an aerobic actinomycete was isolated by Bostroem from similar sources and named A. hominis (sometimes called A. graminis). Bostroem's organism has proved to be a contaminant, however, and it is definitely established that the organism isolated by Wolff and Israel was that observed by Bollinger and Harz and the etiological agent of the disease. These early observations were extended in large part through the work of Wright and Emmons, and summarized by Erikson. 54, 55

Presently, most workers agree that there are two commonly encountered species which cause the disease syndrome "lumpy jaw." A. bovis is the usual cause of actinomycosis in cattle and A. israeli the predominant organism in human infection although occasionally found in cattle. A. eriksonii, a new species, has recently been described in five cases of pulmonary actinomycosis without granule formation.

Morphology and staining. Actinomycosis is essentially a suppurative process characterized by the presence in the pus of yellow granules ("sulfur granules"), the drusen of German writers. These are, in fact, colonies of the bacteria which when examined microscopically are seen to consist of dense rosettes of club-shaped filaments in radial arrangement. The individual rosettes are usually 30 to 40 μ in diameter but sometimes are as large as 200 μ . The minute yellow granules, visible to the naked eye, may consist of a single rosette or may be made up of several. The rosette itself is made up of three kinds of structures: a central core of branching fialments, irregularly disposed but with a general radial

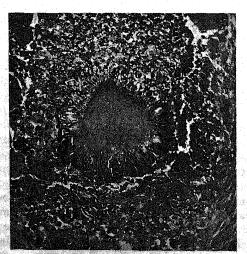
The Pathogenic Actinomycetes and Their Diseases

DISEASE	ORGANISM	GEOGRAPHIC DISTRIBUTION
Actinomycosis	Actinomyces israeli (man) Actinomyces bovis (cattle) Actinomyces eriksonii	Ubiquitous
Nocardiosis (pulmonary and systemic)	Nocardia asteroides Nocardia brasiliensis	Ubiquitous Mexico, South America Africa, India
Mycetoma (actinomycotic)	Streptomyces madurae Streptomyces somaliensis Streptomyces pelletierii	Ubiquitous Africa, Brazil, Mexico Africa, South America
Erythrasma	Corynebacterium minutissima	Ubiquitous
Cracked heel	Nocardia keratolyticus	India, United States
Trichomycosis axillaris	Corynebacterium tenuis	Ubiquitous
Epidemic eczema	Dermatophilis congolense	Australia, Africa, United States

arrangement; refringent, club-shaped bodies at the periphery radially arranged; and spherical coccus-like bodies. The granules may be crushed and examined in fresh preparations in which the clubs may be plainly seen, or a stain such as eosin may be used which colors the sheath of the clubs. The filaments are gram-positive, and the stain is useful for tissue sections; hematoxylineosin is quite satisfactory for sections. The organisms are not acid-fast.

The filaments of the central core are branched, are often curved, sometimes spirally, and are thickly interlaced in a network of mycelium. The individual filaments have a granular appearance and, particularly in older granules, segmentation and fragmentation are common, giving the filaments the appearance of chains of cocci. The individual filaments in these granules, as well as the filaments of Nocardia species and the various agents found in actinomycotic mycetoma, have an average diameter of $1~\mu$, or about the diameter of Escherichia coli. The filaments in tissue of fungus origin and the filaments in granules of mycotic mycetoma are about $4~\mu$ in diameter. This is a convenient method of assessing the category





olikadiki latta (111 merena mendelengak

Figure 185. Actinomycosis (A. israeli) granule in lung. Note the ray-like arrangement of club cells around the central granule. × 300. (Rosebury, Epps, and Clark.)

of disease with which one is dealing. Appropriate antibacterial or antimycotic therapy can be instituted before the specific etiological agent has been isolated and identified.

The club-shaped bodies at the margin of the granule are conspicuous by their high refringency and general structureless, homogeneous appearance. They are pear-shaped swellings of the terminal ends of the filaments and arise as distinct transformations of these. In young colonies the hyaline substance of which the clubs are composed is soft and may be dissolved in water, but as the age of the colony increases, the clubs become of firmer consistency. Their formation appears to be associated with the resistance of the tissues; when resistance to invasion is slight, they are absent, filaments alone being found. Clubs are, as a rule, more common in bovine than in human lesions.

The coccus-like bodies reported by various observers are probably of diverse nature. Such forms may result from the segmentation and fragmentation of filaments; in other cases they may be the ends of clubs appearing in the field of focus of the microscope.

A variety of other microorganisms will be seen in clinical cases of actinomycosis. These include various aerobic and anaerobic micrococci, diphtheroids, gram-negative bacilli, and fusiform bacilli, such as *Bacterium actinomycetem comitans*, which may be a symbiont of some strains of Actinomyces. It is probable that the Actinomyces sp. alone could not induce an infectious process, without the other associated flora.

Morphology in culture. The colonial morphology of A. bovis, A. israeli, and the

saprophyte A. naeslundi grown anaerobically on solid medium is sufficiently distinctive that, with experience and the use of selected physiological characteristics. the organisms can be differentiated from those of contaminating bacteria and from each other. After four to six days' incubation the colonies are often less than 1 mm. in diameter. At this time, all three organisms are usually opaque or dead white, or, rarely, show a slight gray or yellow tinge. A. israeli is generally a rough colony (R form) starting as a mass of branching filaments ("spider" colony or granular colony) with a lace-like border and developing into a lobulated, glistening, "molar tooth" colony. The S variant may be transparent, regular in form, and resemble A. bovis. In broth A. israeli grows slowly, forming a hard, granular, fuzzy-edged colony. A. bovis is generally a smooth (S) form in which colonies are at first dewdrop-like and later smooth, convex and with entire edges. The rare R variant may resemble A. israeli. In broth A. bovis produces a soft diffuse growth. The common mouth saprophyte, A. naeslundi, is generally smooth on agar and rapidly growing, with a diffuse or cloudy appearance in broth. It also grows aerobically after initial isolation; the others are micro-aerophilic after initial isolation. In physiological tests, A. israeli usually ferments xylose and mannitol, reduces nitrate, and does not hydrolyze starch; A. bovis hydrolyzes starch, does not reduce nitrates, and does not ferment xylose or mannitol; A. naeslundi reduces nitrate, but does not ferment xylose or mannitol. All three organisms are separated from anaerobic diphtheroids by their lack of

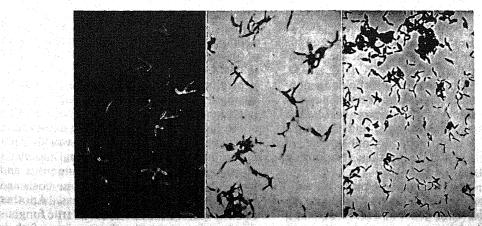


Figure 186. Actinomyces israeli. Left, darkfield. × 900. Middle and right; Gram stains of rough and smooth cultures respectively. × 1200. (Rosebury, Epps, and Clark.)

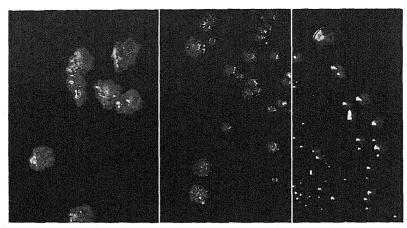


Figure 187. Colonies of Actinomyces israeli on brain-heart infusion agar after six days' incubation. Left, and center, rough "molar tooth" types. × 3. Right, colonies of the smooth type. × 6. (Rosebury, Epps, and Clark.) 188

catalase production. These procedures are discussed elsewhere.7

The essential features of the rosette or sulfur granule have been reproduced in cultures. The smaller colonies are rounded masses of branching and interlacing filaments. As the filaments become older they tend to fragment, and the largest colonies are dense opaque masses of short filaments and rod forms. Clubs are not formed in the usual mediums but only in the presence of blood, serum, or ascitic fluid, and even there develop inconsistently; it is generally agreed that club formation in tissue is in large part a host response. Stained smears of cultures show largely bacillary forms and a few fragments of branching filaments. The bacillary forms may be diphtheroid in appearance, they stain irregularly, and some have terminal swellings; the last are quite unlike the peripheral clubs of the actinomycetous granule.

Serology. By the use of the reciprocal agglutinin absorption method, and subsequently fluorescent antibody. Slack and his associates^{207, 208, 209, 210} separated the actinomycetes into four serotypes: A, B, C, and D. Kwapinski¹³⁵ in a series of articles demonstrated the antigenic relationship of A. bovis and A. israeli to Mycobacterium tuberculosis and various Streptomyces and Nocardia species. The serotypes do not appear to coincide with habitat, and their relationship to immunity is not known.

Pathogenicity for experimental animals. Attempts to infect experimental animals with A. israeli have, in general, been disappointlated animals develop the disease, and the

lesions are limited and benign. Some success has been found by traumatizing the tissue first and including the associated flora with A. israeli. Experimental infection with pure culture has been achieved by Meyer¹⁶⁰ in mice, using hog gastric mucin to enhance the invasiveness of the organism. Others have used repeated injections of the organism in rabbits and guinea pigs with less success. 75 In the experimental disease the essential features of the natural infection are observed, including the formation of tubercle-like nodules and the development of structurally typical granules with clubs.

Pathogenicity for man. 100 Infection with A. israeli during the first part of this century was diagnosed much more frequently than it is today and was, in fact, the most common fungous infection known. There has been a sharp decrease in the number of cases reported in the United States in contrast to the great increase in true fungous diseases. Probably the most important single cause in the decrease of actinomycosis is the widespread use of antibacterial antibiotics. Nichols and Herrel in 1948 reported on the successful use of penicillin in the disease, and this has been a standard therapy since that time. Another important aspect in the decline of infection is the high level of oral hygiene achieved in the developed nations of the world. That this is not the case in emerging nations is attested to by the continued numerous and increasing case reports of actinomycosis and related diseases in these areas. Another relative factor in the increase in true fungous ing in that only a small proportion of inocu- diseases is a better understanding of their etiology, incidence, and clinical course, as

695

well as the improved diagnostic methods available (when used).

NOCARDIOSIS

The disease in man differs in minor respects from that in cattle. Actinomycotic infections of the bone are relatively less frequent, the disease being confined to the softer parts in most cases. There is usually a lesser production of new tissue and a more extensive softening and suppuration. The disease falls into three clinical types. About 60 per cent of the infections are cervicofacial, and this type is often associated with dental defects or accidents and is a chronic. localized form of the disease which is relatively benign and usually susceptible to treatment. Some 14 per cent of the cases are thoracic infections, and 8 to 18 per cent are abdominal infections³⁴ in which the primary lesion is often in the appendix; in these types prognosis is poor. Draining sinuses are usually found in all types, and abcesses are frequently observed in the liver at autopsy. Generalization by hematogenous spread is occasionally seen and is relatively more common in man than in cattle. Meningitis, endocarditis, genital (both male and female) infections, and a syndrome-like mycetoma have been reported. The characteristic granules with clubs are usually found in the pus but may be absent occasionally in draining sinuses, especially when the infection has spread rapidly, particularly in meningitis and empyema. These latter types of disease may terminate fatally in a few weeks through secondary infection or formation of emboli, or may drag along in a chronic form for many years; spontaneous healing has been observed.

Immunity. Agglutinins, precipitins, and complement-fixing antibodies have been demonstrated in the serum of patients with actinomycosis. There is little or no evidence that they are involved in combating the disease or protecting the individual against the disease. Active defense against the organism is probably on a cellular level, and it has been suggested that the "clubbing" of the organism seen on the periphery of the granule represents a response to the cellular defense of the host.

Isolation and diagnosis. 82, 85, 100 As indicated earlier, the demonstration of the typical actinomycetous rosette or sulfur granule in the tissue or pus of a specimen is sufficient to establish a diagnosis of actinomycosis in man. Actinomycosis in cattle may be confused with actinobacillosis; the two are readily differentiated by examination of a

gram-stained smear for the presence of gram-positive diphtheroid-like fragments of Actinomyces filaments or of gram-negative actinobacilli. As may be inferred from the foregoing discussion, animal inoculation is useless as a diagnostic method. Cultivation of the bacteria is often, though not always, successful.

The procedure recommended by Wright for the isolation of A. israeli in culture has been widely used. Granules, preferably from a closed lesion, are thoroughly washed in several changes of sterile water or broth and then crushed between sterile glass slides. The material is inoculated into liquid (40° C.) 1 per cent dextrose agar in test tubes filled to a depth of 6 to 8 cm. After incubation at 37° C. for four to eight days, characteristic colonies develop, in greatest numbers in a narrow zone 5 to 12 mm. below the surface. These may be isolated from the shake culture in the usual way, washed in sterile water or broth is there is reason to suspect bacterial contamination, and subcultured in deep tubes of dextrose agar. Even in the absence of contamination the method is not particularly satisfactory and frequently fails.

Rosebury, Epps, and Clark 188 have shown that abundant and characteristic growth occurs on the surface of enriched agar mediums containing dextrose and incubated under anaerobic conditions in the presence of carbon dioxide. They recommend brainheart infusion containing 2 per cent agar. The granule, exudate, or other material is streaked serially on plates of the medium by means of a sterile bent glass rod, and the plates are incubated four to six days under anaerobic conditions in the presence of 5 per cent carbon dioxide. By this method A. israeli can be isolated even in the presence of heavy bacterial contamination.

NOCARDIOSIS²³³

Several species of aerobic actinomycetes are capable of causing human disease. ¹⁹⁸ All of these organisms are soil organisms and infection is endogenous. A possible exception to this is *Nocardia asteroides*, the etiological agent of nocardiosis. The organism has been isolated frequently in nature from soil, from infections in small animals and fish and as transient flora of the skin. Hosty et al., ¹¹⁰ in surveying the sputa from tuberculosis patients, examined over 85,000 specimens and found *N. asteroides*

in 175. A diagnosis of nocardiosis could not be established in these cases. Thus, the Nocardia might be considered as a saprophyte or minor secondary invader in these cases. Since these patients all had some lung disease, it is difficult to extrapolate to the normal population, as survey studies have not been adequate. It is suggested, however, that *N. asteroides* may be at least a transient member of normal pulmonary flora. Although pre-existing lung disease may favor a frank infection by the organism, this is not a necessary prerequisite.

The genus Nocardia was erected by Trevisan in honor of Nocard. Nocard had earlier described an aerobic, partially acidfast, branching bacillus from cattle as the causative agent of a disease called farcy. Some confusion exists concerning the name Nocardia farcinica, as used by Trevisan. Gordan and Mihm⁹³ suggest that N. asteroides be considered the type species of the genus and N. farcinica be reduced to synonomy with it. The name N. asteroides was first used by Blanchard in 1895 in referring to the Cladothrix asteroides of Eppinger. The first description of the disease in human patients was made in 1890 by Eppinger, who isolated an aerobic, branching, fungus-like organism from pulmonary and central nervous system lesions.

The genus Nocardia formerly contained various species isolated from the clinical entity mycetoma. Most of these species have been placed in the genus Streptomyces by MacKinnon¹⁴⁸ and are discussed under the heading Mycetoma. The remaining species, *N. brasiliensis*, is a common cause of mycetoma and is described with the other agents. This organism can also cause systemic nocardiosis.⁹²

There is a broad spectrum of disease elicited by N. asteroides infection. The variation probably reflects intensity of exposure to the agent and route of infection. Pulmonary disease is by far the most common manifestation of infection and varies from single lesions and scattered infiltrations to lobar consolidation and cavitation. The disease picture resembles that in tuberculosis, histoplasmosis, or other mycotic infections. Candida albicans superinfection may occur in this as in other mycotic diseases, and it is necessary to search thoroughly for an underlying agent.

Hematogenous spread of the infection may result in a secondary infection of the brain. Occasionally, there may be minimal infection in the lung, and the presenting symptoms are extrapulmonary. The kidneys, spleen, liver, and adrenals may be involved; however, in contrast to *A. israeli* infection, bony involvement is rare. The rare reported cases of *N. asteroides* mycetoma are difficult to assess, as there is no clear differentiation from *N. caviae*.⁸³

Serology and immunity. A skin test using a ground mycelial extract, nocardin, has been used with varied and limited success. An early preparation called asteroidin was reactive intradermally in infected experimental animals. Circulating antibodies have been reported, but their role in disease has not been assessed.

Pathogenicity for lower animals. The most frequently involved domestic animal is the dog. Similar types of pathology are seen in both animal and human infections. Cattle are also involved with infection, some in epidemic numbers. The relation of farcy of cattle to *N. asteroides* has been discussed. The organism also infects fish.²¹⁷

Isolation and diagnosis. In gross examination, lesion material cannot be differentiated from that in other infections of pyogenic bacteria. The tissue reaction, like that to A. israeli, is of the pyogenic type, acute or chronic suppurative with neutrophilic infiltration. The organism is best seen in tissue sections stained by the Brown and Breen modification of the Gram stain. The Gomori

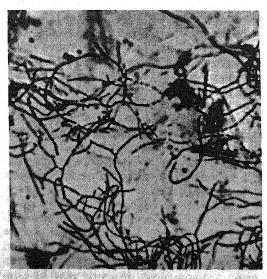


Figure 188. Nocardia asteroides. Gram stain of sputum. Note the long branched mycelium. \times 900.

697

methenamine stain is also useful. PAS and H and E are not helpful. Fine branching filaments, 1 μ in diameter, are seen coursing through the tissue. There is a lack of agglomeration or granule formation, as seen in A. israeli infection. Otherwise it is not possible to differentiate the two except by culture.

The organism grows well but slowly on all laboratory mediums and is usually not killed by the digestion procedure used on sputa for culture of tubercule bacilli. The classic colony is glabrous, wrinkled, folded, and bright orange. Variations from slick yeast colonies to dry powdery colonies with abundant aerial mycelia have been described. The color range recorded includes pink, lavender, salmon, white, buff, and brown.⁹³

Prognosis of the disease depends on early diagnosis and treatment. There has been a notable lack of success in established infections, especially those with extrapulmonary lesions. Sulfonamides are the favored drugs, especially sulfadiazine. Streptomycin, Aureomycin, and penicillin have also been used.

OTHER ACTINOMYCETOUS INFECTIONS

Erythrasma. This disease was first described by Burchardt in 1859, and the term erythrasma was used by Barensprung in 1862. Skin scales examined by these authors showed delicate filaments which were believed to be of fungous origin. They named the organism Microsporum minutissimum. In skin scales and on culture the organisms appear as gram-positive rods and filaments sometimes with granules. The scaling infected area of the patient and the colonies of the organism, when grown on tissue culture medium number 199 agar, show a coral red fluorescence which aids in its diagnosis. The disease itself is characterized by a punctate to palm-sized, well-circumscribed, maculopapular rash on the epidermis. The color varies from light brown to red or reddish brown. The advancing border is serpiginous and erythematous. The lesion is greasy looking and is covered with small furfuraceous scales. The most common form is genitocrural in the male, although other areas and other groups may be affected. Sarkany, Taplin, and Blank 195 found that infection of the toe web may occur in about one-fourth of the population. Systemic

erythromycin is the drug of choice. At present the organism is called *Corynebacterium minutissimum*.

Cracked heel (pitted keratolysis). This disease, also called keratolysis plantare sulcatum, was first described in India, but recently Zaias, Taplin, and Rebell²³⁷ have shown it to be of world-wide distribution. The etiological agent remains obscure but appears to be an actinomycete, either Nocardia or Micromonospora keratolytica. The bacteria appear to have a lytic action on the horny layer of the epidermis, and the disease is characterized by solution of the thick horny skin of the plantar surface in grooves. The skin cracks along these grooves to form deep fissures on the heel and in the thick sodden skin between the toes. These fissures extend through the corium to the subcutaneous tissues. Secondary infection is common. A similar disease, ulcus interdigitale, may occur on the toes. Treatment consists in good hygiene and the wearing of shoes.

Trichomycosis axillaris. This disease is characterized by yellow (flava), red (rubra), or black (nigra) concretions on the shaft of axillary or pubic hair. Perspiration in the affected area may be accordingly discolored and stain the clothing, this being the most common presenting condition. There is no other discomfort to the patient. Recently, an investigation of 100 consecutive patients revealed 28 cases of the flava variety without the patients' being aware of the infection.35 The disease may be more common than formerly considered. The organism has been named Corynebacterium tenuis and the flava variety will fluoresce. It is probable that a number of organisms are involved or are capable of producing the symptoms. Treatment includes depilation, alcoholic formalin solution 1 percent, and sulfur oint-

Streptotrichosis (epidemic eczema, contagious dermatitis). This disease was first described by Van Saceghem in 1915 as a skin disease of cattle. He named the etiological agent Dermatophilus congolense. Other isolates from cattle, sheep, deer and horses have been given different names, but Gordon⁹¹ concludes they are variations of a single species. The organism was first reported in the United States in 1961 and since has been reported from cattle, horse, deer, and man in Texas, Iowa, and New York. It now appears that the organism is of world-wide distribution, Roberts¹⁸⁵ be-

lieves the species to be a natural parasite of the epidermis of sheep.

Dermatophilis congolense grows in culture as a moist, lumpy, yellowish colony and microscopically as a branching mycelial mass in which the hyphal elements enlarge and go through a series of longitudinal and transverse divisions to form a motile coccal form. The motile stage formation is stimulated by low temperature, restricted nutrients, excess aeration, and moisture. The organism is a true epidermal parasite, normally not penetrating the dermal-epidermal junction. The disease in sheep is characterized by erythematous, exudative, scaling lesions which develop into pyramidal scabby masses. This condition is sometimes called lumpy wool. The organism is spread by contact and ectoparasites and is favored by cold, damp conditions.

MYCETOMA (Maduromycosis)27, 148

Mycetoma (fungous tumor) is a clinical syndrome which may have either a fungal or bacterial etiology. Though the mode of infection, type of tissue reaction, and general course of disease are similar, the choice of treatment depends entirely on knowing whether the infection is bacterial or fungal in origin. The first cases of this infection were reported from southern India (1842 et seq.) and became known as Madura foot. It is mainly a disease of tropical climates and appears usually in persons who do not wear shoes. The infection is frequently seen in temperate areas about the Mediterranean. in North Africa, Greece, and Italy. It also regularly occurs in Mexico, Central and South America, and the Caribbean Islands. Cases are sporadically seen in Europe and the United States. Most of the etiological agents are world-wide in distribution, but some are more common in one area than another, such as Streptomyces somaliensis in North Africa or Nocardia brasiliensis in Mexico.

The disease usually affects the foot, occasionally the hands, and more rarely other parts of the body. There are exceptions, as Nocardia brasiliensis is commonly associated with pulmonary infections. The organism presumably enters by traumatic implantation into tissue. There is some evidence that it may remain latent for some time. even a period of years, and become active following injury. The part first involved.

usually the sole of the foot, shows a small subcutaneous swelling which slowly enlarges and softens to become phlegmonous. It ruptures to the surface, sinus tracts form. and the process burrows into the deeper tissues, producing swelling and distortion of the foot; the bones may or may not be extensively involved. Numerous eminences are found on the surfaces, each the orifice of a sinus. The discharge is a viscid, slightly purulent, often foul-smelling fluid containing granular particles up to 1 mm. in diameter. The presence of these grains separates the mycetomas from pseudomycetomatous conditions observed in yaws, sporotrichosis, etc.

Examination of the granules will reveal whether the disease is one of the two types of mycetoma or is the mycetoma-like disease, botryomycosis, caused by Staphylococcus aureus. If the disease is mycetoma, careful examination of the granule (and subsequent confirmation by culture) will reveal which of the two groups is the etiological agent. The most obvious clinical difference in the mycetomas is the color of the grains present in the discharge. These may be white, yellow, red, or black. The grains or microcolonies are composed of mycelial filaments of the organism. One of the agents, S. madurae, has been shown to elaborate a powerful collagenase¹⁸² which may be involved in infection. 183 If the granule is composed of very thin $(1 \mu \text{ in diameter})$

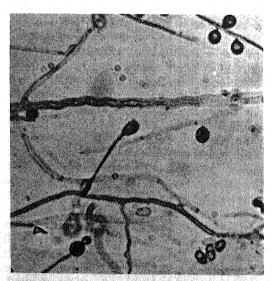


Figure 189. Allescheria boydii. Culture mounted in lactophenol-cotton blue solution. Single spore on elongate conidiophore. × 400.

ARABETHEOR SHE COSTONED

Mycetoma
Actinomycotic
of
Organisms

Delicate branched myce- lium, not acid-fast	Same as above. Conidia may be seen.	Same as above. Conidia are usually not seen.	Short irregular rods, occasional branched mycelium. Conidia seen, Partial acidfast.	Same as above. Note: Actinomyces israeli an anaerobe, may also rarely cause a yellow grain mycetoma.
Fast growth, cream, smooth, glabrous	Slow growth, cream to brown, very wrinkled, flaky	Slow growth, small granules, red colony	Rapid growth, pale tan, glabrous to wrinkled, dry	Rapid growth, classically bright orange, folded, glabrous; variable
1.5 mm. D., clubs	1.25 mm. D., no clubs	1 mm. D., no clubs	1 mm. D., clubs	1 mm. D., clubs
White, irregular	Yellowish, compact, round, hard	Garnet red, lobulated, hard	Yellow, soft, lobulated	Grains rare, soft, yellow, irregular
Usually not successful unless dose is very high (50-200 mg.) ¹⁸³	Not successful	Not successful	Abscesses and grains ¹⁴⁹ in mice, guinea pigs	Abscesses, no grains, usually fatal in animals
Streptomyces madurae	Streptomyces somaliensis	Streptomyces pelletierii	Nocardia brasiliensis	Nocardia asteroides
	Usually not successful White, 1.5 mm. D., Fast growth, cream, smooth, unless dose is very irregular clubs glabrous high (50-200 mg.) ¹⁸³	Usually not successful White, 1.5 mm. D., Fast growth, cream, smooth, unless dose is very irregular clubs glabrous high (50–200 mg.) ¹⁸³ Not successful Yellowish, 1.25 mm. D., Slow growth, cream to brown, compact, no clubs very wrinkled, flaky round, hard	Usually not successful White, 1.5 mm. D., Fast growth, cream, smooth, unless dose is very irregular clubs glabrous glabrous high (50–200 mg.) ¹⁸³ Not successful Yellowish, 1.25 mm. D., Slow growth, cream to brown, compact, no clubs very wrinkled, flaky round, hard Garnet red, 1 mm. D., Slow growth, small granules, lobulated, no clubs red colony hard	Usually not successful White, 1.5 mm. D., Fast growth, cream, smooth, unless dose is very irregular clubs glabrous glabrous high (50–200 mg.) ¹⁸³ Not successful Yellowish, 1.25 mm. D., Slow growth, cream to brown, compact, no clubs very wrinkled, flaky round, hard Garnet red, 1 mm. D., Slow growth, small granules, lobulated, no clubs red colony hard no clubs red colony hard lobulated, 1 mm. D., Rapid growth, pale tan, glain mice, guinea pigs lobulated clubs brous to wrinkled, dry

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Organisms of Mycotic Mycetoma (Tissue Dimorphic Fungi)

MICROSCOPIC CHARACTERISTICS	Unicellular, conidia, born singly on long condiophores. Cleistothecia has eight spored asci. Imperfect stage called Monosporium apiospernum.	Numerous chlamydospores, rare conidia from phialides. Infection in mice 162	Same as above.	Toruloid chains of budding cells and conidia from long phialides. May cause chromoblastomycosis or subcutaneous mycosis.
COLONIAL CHARACTERISTIC	Rapid growing, fluffy mouse- fur color	Very slow growing, apricot to white; sometimes black sclerotia	Very slow growing, tan-gray, velvety; black sclerotia	Slow growing, black, gla- brous or velvet
SIZE	0.5 mm., round	1 mm., round, lobed	1 mm., round, lobed	Irregular
RAIN COLOR	White	Black	Black	Black
SPECIES GRAIN	Allescheria boydii	Madurella mycetomi	Madurella grisea	Phialophora Jeanselmi

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intertwined mycelial elements, often with peripheral club cells, it is bacterial (actinomycotic mycetoma); mycelial elements more than 1 μ in diameter showing individual cells, septation, and usually chlamydospore production are found in fungous (mycotic mycetoma) infections. Actinomycotic mycetoma is treated with the usual antibacterial antibiotics such as penicillin, tetracyclines, sulfonamides, and diaminodiphenyl sulfone. Much less success has been observed in the treatment of mycotic mycetoma. Intravenous amphotericin B has been used, with remission of the disease in some cases. Iodides are still used with some success.

The disease is usually local, secondary abscesses seldom if ever occur, and the internal organs are probably never affected. In cases resistant to therapy or with extreme tissue destruction, surgical removal of the infected tissues, usually involving amputation, is necessary and curative. Occasionally the organisms associated with mycetoma

may be involved in quite different infectious processes, such as Allescheria in prostate¹⁵⁹ and eve⁵⁶ infections.

Laboratory diagnosis. As indicated above, the mycetomas are characterized by the presence of granules in the discharge. The grains may be hard and are usually treated with strong (20 per cent) sodium hydroxide to dissolve the pigment and debris and to permit examination in a wet mount (unstained) for the presence of tangled mycelial filaments. Clubs may be observed in actinomycetous infections, and thallospores (sclerospores), heavy-walled homogeneous structures, are found in Madurella and other fungous infections. For differentiation and identification, culture is necessary on enriched meat media (BHI agar) for actinomycetes, and Sabouraud's agar in the case of fungal etiology. Serology has been little used in mycetomas. Usually no antibody titers can be detected except in the more extensive infections, as occurs in N. brasiliensis pulmonary and systemic involvement.

The Pathogenic Fungi

The fungi proper, or Eumycetes, are quite distinct from the true bacteria, in size, cell structure, nuclear structure, and chemical composition. Differences in cell wall composition and nuclear structure were previously discussed, under the section on actinomycetes. Though essentially a single-celled organism, in some fungal species the cells may show various degrees of specialization. The simplest morphological form is the single-celled budding yeast; elongation of the cell without separation of new-formed cells results in a thread-like hypha; an intertwined mass of hyphae is called a mycelium. The mycelial mat is known as a thallus and, in most of the fungi discussed here, is a loose network of hyphae. In some higher forms, hyphae are cemented together to form large. structurally complex fruiting bodies such as the mushrooms and puffballs, which may weigh more than 60 pounds. Even though attaining such great size, all fungi are still primitive organisms: any separated single cell from a 60-pound puffball can regrow the entire structure. This ability separates the fungi, protozoa, and algae from higher forms such as trees and mammals.

Two main structural types of mycelium may be distinguished. In one of these the

cells making up the hyphae are not separated by cross-walls or septa, making possible the characteristic flowing of protoplasm through the multinucleate structure. Such a structure is said to be nonseptate or coenocytic. Certain of the algae are of similar structure, and the fungi thus characterized are called phycomycetes or algae-like fungi. In the majority of fungi, however, the hyphae are septate, each cell separated from the other by crosswalls. The septations have "holes" in them so that there may be free flow of cytoplasmic material. Nuclear migration is possible in the ascomycetes, which have a large pore in the intracellular septa. A special structure, comprising the dolipore and parenthesome, prohibits nuclear migration in the basidiomycetes. Each basidiomycete cell contains two dissimilar nuclei (dikaryon), one derived from one parent or mating type and the other from the other parent. The mycelium of this latter group may also show a special bridge structure connecting one cell to the cell next in line. This is called a clamp connection and is involved in nuclear transfer to a newformed cell. Thus these three main classes of fungi may be distinguished in part on the basis of the structure of the mature nonsporulating hyphae, though this is not one of the main characteristics upon which their differentiation is made.

The mycelium is further differentiated into two general types which differ in function. One of these, the vegetative mycelium. consists of masses of hyphae within the colony, adjacent to and growing into the substrate, and is concerned with the assimilation of food materials. Fragments of such mycelia will reproduce if transferred. The other type, the reproductive mycelium, usually extends into the air to form an aerial mycelium and gives rise to reproductive bodies or spores. The mode of spore formation and the structure of the spore and sporebearing elements are the characteristics by which the fungi are differentiated, classified, and identified.

In addition to sporulation and the vegetative growth of hyphae, many species of fungi reproduce by the separation of cells, known as oidia, from any part of the mycelium. These vegetative reproductive forms may give rise to new mycelium or reproduce themselves by budding like the yeasts, according to the environment in which they

are placed.

Spore formation. Two kinds of spores are to be distinguished, the sexual spores, which are produced by the fusion of two cells which may or may not differ, and the asexual spores, which arise by differentiation of the cells of the spore-bearing hyphae without fusion. If a sexual spore is produced only with a nucleus from another mating type, the fungus is said to be heterothallic. If any nucleus from within the same thallus will serve in forming sex spores, the fungus is homothallic. Fungi for which both sexual and asexual types of spore formation are known are "perfect fungi" and may be further differentiated on the basis of differences in the fruiting body that results from sexual fusion and differences in type, size, color of sexual cells, and other morphological characteristics. The three classes of fungi, based on the method of sex spore formation, are Phycomycetes, Ascomycetes, and Basidiomycetes. A fourth group is the formclass Deuteromycetes, or Fungi Imperfecti, for which no "perfect" or sexual state is known.

Sexual spore formation. The most common of the Phycomycetes are species of Mucor and Rhizopus. These molds produce a sexual spore known as a zygospore by the fusion of neighboring filaments of the same or different plants. The Ascomycetes, which include a number of genera as well as certain strains of Penicillium and Aspergillus, and also the yeasts, form sexual spores known as ascospores because they are contained in an ascus or sac. Their formation is simplest in the yeasts, in which two contiguous cells fuse by means of minute tube-like processes. The nuclei unite, and the resulting single nucleus divides several times to give four or eight daughter nuclei. Reserve material accumulates about each nucleus, a spore wall is formed, and the cell containing this is the ascus. In most of the Ascomycetes the process is somewhat more complex, and the cells which fuse may be distinguishable and are termed the oögonium and antheridium. The cell resulting from this fusion gives rise to new hyphae. It then goes through a complex structural development called crozier formation, with the next to last cell being binucleate. These nuclei fuse and then divide to form the ascospores, i.e., two nuclear fusions are involved in the entire process.

The basidiospores, the reproductive unit of the Basidiomycetes, are also formed by a complex process including clamp connection formation, by which one nucleus of a mated pair travels from one cell to a newly formed cell, the other nucleus going by the "center road" through the middle of the cell. The nuclei finally fuse in a club-like structure called a basidium and give rise to four externally attached basidiospores, two of the cells being the mating type of one parent and two the mating type of the other parent. Basidia line the gills of the mushrooms, are found in crypts inside puffballs, and line the pores in Boletes. So far, none of the Basidiomycetes has been shown to be pathogenic. The Ascomycetes include Piedraia hortae (black piedra), Allescheria boydii (mycetoma), and the dermatophytes (usually known by their imperfect name). The Phycomycetes are represented by Mucor, Rhizopus, Basidiobolus, etc. Many pathogenic fungi have no known sexual phase and are classed as Fungi Imperfecti.

Fungi Imperfecti. The "imperfect fungi" or Fungi Imperfecti (Deuteromycetes) are sometimes called hyphomycetes; they make up the fourth group of Eumycetes. The group is necessarily provisional, and various species of fungi are removed from it from time to time as their sexual phases are discovered. The classification of the organisms is based on asexual spore-type formation, color, shape, size, etc. Often an organism will be given a name based on its asexual characteristics before its sexual stage is known. Later, when a sexual stage is discovered, a name descriptive and taxonomically meaningful will also be given to it. This sometimes leads to two names for the same organism. It may be confusing at first, but it is taxonomically legal, for example, for the dermatophyte asexual phase to be Microsporum gypseum and its perfect state to be Nannizzia gypsea. To add to the confusion, the organism we call M. gypseum is the imperfect stage of two species of perfect fungi, N. gypsea and N. incurvata. This emphasizes the concept of Fungi Imperfecti as being simply a convenient descriptive filing cabinet for species waiting to be assigned to meaningful categories if only their sexual mechanisms can be discovered.

Microscopic examination. The methods used in the microscopic examination of the fungi vary somewhat according to the nature of the material and the purpose of the examination. In general, the staining methods so useful in the study of the bacteria are not applicable; the fungi are almost all grampositive and may be found in gram-stained smears, but their morphology is obscured. Wet mounts, unstained or lightly stained, are most informative.

Specimens. Open or draining lesions are almost always so heavily contaminated by secondary bacterial invasion that fungi are very difficult to find; in material from surgically opened lesions they are usually demonstrable though not so numerous as bacteria in corresponding bacterial infections. A simple method to show the yeast cells, mycetoma granules, or mycelial units in pus, exudate, or sputum is to treat the specimen with potassium hydroxide (KOH mount) in the manner described below. In the dermatomycoses, secondary bacterial infection ordinarily does not interfere with the microscopic demonstration of the fungous elements. The dermatophytes live in keratin material exclusively, and specimens should be taken from scrapings of horny layers, tops of vesicles, scrapings from nail plates, and hair.

The material should be mounted in strong (10 to 20 per cent) hot potassium hydroxide—which dissolves or makes translucent the tissue elements and bleaches the pigment in mycetoma granules but ordinarily affects the fungus more slowly—and examined as a wet unstained preparation. Antiformin or lactophenol may be used

instead of potassium hydroxide. Care must be taken to distinguish between spores and fat globules and between mycelium and fibrin strands; a mycelium-like structure ("mosaic fungus") may be formed in some skin scale preparations, presumably from cholesterol. Fungi may be demonstrated in tissue sections, as the walls of abscesses and granulomatous tissue, by the Unna-Pappenheim (methyl green-pyronine). Hotchkiss-MacManis (periodic acid-Schiff), Gridley (a modified PAS), or Gomori (methenamine silver) stain. Gomori's stain is recommended for finding the few organisms in a large specimen, and Gridley's for distinguishing the detail of fungus structure. Actinomycetes are not stained by these procedures; for these organisms the modified Gram stain of Brown and Breen is recommended

Cultures. A bit of growth is removed from the colony, teased apart in a drop of water, and examined as a wet preparation. While the various structures may be seen, the arrangement of the elements is seriously disturbed. Slide cultures show the structure and arrangement of the growth and may be made into permanent mounts. These are especially recommended for identification of dermatophytes; they are not recommended for Coccidioides, Histoplasma, etc.

Cultivation. 7 Most of the fungi grow rapidly, but the pathogenic forms usually grow relatively slowly: three or four weeks' incubation may be necessary. Their morphology is markedly affected by the type of medium on which they are grown. In general, they show no unusual nutritive requirements and grow readily on all the usual bacteriological mediums, especially if a sugar is added. Many molds tolerate a high acidity and may be cultivated on tartaric acid-dextrose nutrient agar, on which bacterial growth is inhibited. Most grow well on modified Czapek-Dox medium (a glucosenitrate synthetic medium), which is reproducible and has been widely used as a "standard" agar for descriptive purposes. Sabouraud's medium, a peptone-maltose agar, is perhaps the most widely used medium in medical mycology for the isolation and maintenance of cultures, especially the dermatophytes.

In present laboratory practice, antibiotics are added to make the medium selective for a particular group of organisms. For dermatophytes and the mycelial stage of the thermal dimorphic fungi, Sabouraud's medium

which chloramphenicol (against bacteria) and cycloheximide (Actidione), which suppresses most contaminant fungi, is very useful. Some pathogenic fungi will not grow on Actidione medium, e.g., Candida tropicalis, the yeast stage of Histoplasma and Blastomyces, and Cryptococcus neoformans; all actinomycetes are inhibited by the Chloromycetin. If these organisms are suspected, other culture methods must be used.

The fungi to be discussed in this section will be arranged as to the clinical location of their site of infection. 90 The categories are as follows:

Superficial mycoses: Only the outermost layers of skin or hair are involved and there is practically never any host reaction to the parasite, as in piedra and tinea versicolor.

Cutaneous mycoses: The infection is limited to the superficial layers of the skin with little or no invasion of living cells. There may be significant, and occasionally severe, host reaction to the presence of the organism. The dermatophyte and dermal Candida infections are included.

Subcutaneous mycoses: In this category are diseases in which the organism has invaded or has been implanted into subcutaneous tissue. Usually the infection is limited to the site of entry and a long clinical course may ensue. Chromoblastomycosis, mycetoma, subcutaneous phycomycosis, and sporotrichosis are examples.

Systemic mycoses: The most dangerous and often fatal disease-producing organisms are in this category. Entrance of the organism is usually through the lung, and the disease may spread to other parts of the body. The diseases of the thermal dimorphic organisms (histoplasmosis, blastomycosis), systemic candidiasis, coccidioidomycosis, cryptococcosis, etc., are discussed.

Rare mycoses: The mycoses that are not often encountered and the organisms not usually considered pathogenic are included. These may be soil organisms re ponding to an unusual environment after accidental implantation or invasion of a debilitated host. Such diseases as aspergillosis, phycomycosis, and cladosporosis are considered.

THE SUPERFICIAL MYCOSES

TINEA VERSICOLOR (Pityriasis Versicolor)

This is a common, world-wide fungous infection. It is most common, however, in

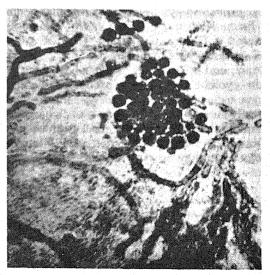


Figure 190. Skin scale from tinea versicolor. Short branched mycelium and small yeast-like cell are diagnostic of this disease. Methenamine silver: × 400.

tropical areas. The disease is characterized by yellow to brown patches or continuous scaling over the trunk and occasionally the legs, face, and neck. The affected area fluoresces a golden vellow when irradiated with a filtered ultraviolet lamp, "Wood's light" (peak at 3560 Å). Susceptibility is probably genetic and may be related to the rate of epidermal growth and desquamation. High endogenous or administered cortisone levels may also predispose to the disease.123 The etiological agent was called Mallassezia furfur in the older literature. There is considerable evidence that the organism is the lipophilic yeast Pityrosporum orbiculare. 131 It will not grow on ordinary mediums, usually requiring some lipid additives. Diagnosis is easily made by examining the scales in a KOH mount. Short, thick hyphal elements and associated round forms are seen. Successful treatment includes selenium sulfide shampoo and Whitfield's ointment. The infection usually recurs.

TINEA NIGRA

The disease tinea nigra makes itself manifest as dark blotches on the palms of the hands and rarely elsewhere on the body. In a KOH mount the organism appears as brown, branched hyphae, 2 to 3 μ in diameter. Though most common in the tropics, occasional cases occur in the United States. The etiological agents are members of the genus Cladosporium, which

is probably synonymous with the genus Hormodendrum. C. werneckii is commonly found in Central and South America, while C. mansonii is encountered in India, tropical Asia, and Africa. The colonies grow slowly on cycloheximide agar, producing a dark, greenish black, moist colony. Microscopically numerous, dark, one- and two-celled, budding conidia are seen on dark mycelium. Keratolytic fungicides, such as Whitfield's ointment, may be used in treatment.

PIEDRA

This disease is a fungous infection of the hair, characterized by nodules on the distal shaft. Two types are encountered. White piedra, in which soft light-colored nodules form, is principally found in temperate regions. The etiological agent, Trichosporon cutaneum, is sensitive to cycloheximide but will grow on most other mediums. It produces a soft, creamy, rapidly growing colony which becomes yellowish gray. Microscopically, it is composed of hyaline septate mycelium that fragments into oval arthrospores. T. cutaneum is separated from other members of the genus by its lack of fermentation of sugars, its lack of growth on potassium nitrate, and its carbon assimilation pattern. Black piedra is a hard, black or brown nodule caused by the ascomycete,



Figure 191. Black piedra knob on hair shaft. Piedraia hortae. × 100.

Piedraia hortae. This disease is mainly tropical, being found in Latin America, Asia, and Africa. It also infects other primates. The organism grows slowly on cycloheximide agar, producing a greenish to rusty black, hard, raised colony. The colony is composed of dark mycelium and chlamydospores. Asci are rarely seen in culture. Treatment consists in shaving off the hair.

THE CUTANEOUS INFECTIONS (Dermatophytoses)⁵

By far the most common type of fungous disease of man is dermatophytosis (dermatomycosis), a superficial infection of the keratinized epidermis and keratinized epidermal appendages, i.e., the hair, hairsheaths, and nails, the severity of which is dependent for the most part upon the location of the lesion and the species of fungus involved. Though certain other fungi, notably Candida, produce clinically similar disease, a more or less homogeneous group of fungi, the dermatophytes, is responsible for the great majority of cases. The ability of these microorganisms to invade and parasitize the cornified tissues is closely associated with, and dependent upon, their common physiological characteristic: the utilization of the highly insoluble scleroprotein keratin. The utilization of keratin is biologically rare and is shared by the dermatophytes (family Gymnoascaceae) with only a few species of saprophytic fungi (notably the family Onygenaceae) and certain insects including the clothes moth (Tinea), the carpet beetles (Dermestes), and the biting lice (Mallophaga).

Though the various species of dermatophytes produce infections that are clinically characteristic, on the one hand single species may produce different types of disease and, on the other, infections that are very similar or essentially identical may be produced by different species. Furthermore, other conditions such as chemical dermatitis, neurodermatitis, and certain types of allergy may closely simulate dermatophytosis, but do not, of course, respond to treatment with fungicides. Demonstration of the causative fungus by direct microscopic examination of pathological material or by isolation and culture is, therefore, highly desirable, especially in all cases presented for treatment.

The antibiotic griseofulvin has proved to be a highly effective chemotherapeutic agent for the treatment of the dermatomycoses, 106, 178, 179, 189, 238 but cure commonly requires prolonged (three to 10 weeks) administration by mouth. There may be severe side effects from the use of this drug. 191 The antifungal effect of the antibiotic is reflected in morphological changes in the infecting fungus, both *in vivo* and *in vitro*, and may be involved in utilization of keratin. A new drug, tolnaftate (Tinactin), has had promising results in most infections, except those of the hair and nails.

The dermatophytes were until recently classed as Fungi Imperfecti. Through the work of Stockdale,²²⁰ Gentles, and others, it has been demonstrated that many have a sexual stage (and hence a perfect state name). This observation was first made by Nanizzi in 1927 but was ignored. Consequently, many of the organisms are known by two names. In clinical dermatology the older imperfect stage name is still in common usage and probably will remain so. Most of the organisms shown to have a perfect state are heterothallic and will not show ascus formation except when grown with an opposite mating type. Even then, cleistothecia formation usually may occur only on mediums with a mixture of keratin and soil. A list of the known dermatophytes and their perfect stages is given below. Many related organisms have been isolated from the soil, but as yet have not been isolated from lesion material or only rarely.67

These organisms differ from most other

pathogenic fungi in that the cells are multinucleate, usually containing four to six nuclei, and division is amitotic. Arthrospores, chlamydospores, and the individual cells of fuseaux (macroconidia) are also multinucleate, but aleuriospores and microconidia contain but a single nucleus. The group is a homogeneous one, immunologically as well as morphologically and physiologically. Only recently have some physiological differences been found which are an aid in identification. These will be discussed later.

Differentiation of genera and species. 77, 141 David Gruby in 1841 described the fungal etiology of favus. Culturing the fungus from an infected lesion, he reproduced the disease by inoculation into normal skin. After this time, many fungi were isolated from similar lesions. Some were the responsible agents, many were contaminants, and practically all were given different names without regard to proper taxonomic procedures. Some order was brought about by the work of the French dermatologist Sabouraud. The classification of Sabouraud, presented in 1910 and somewhat modified by him later, is the most generally used. The system was extensively revised by Emmons⁴⁹ and Conant, who reduced to synonomy many varieties of the same species. Presently, work on the ascigerous state and physiological differences will help in establishing species lines. As of now, we are still dependent on differences in colonial and microscopic morphology, pigment, spore types, etc.

Colonial morphology is readily altered

The Ascigerous States of Dermatophytes and Related Species

IMPERFECT STATE	USUAL HOST	PERFECT STATE
Microsporum gypseum	Man, animals	Nannizzia gypsea Nannizzia incurvata
Microsporum fulvum	Man, animals	Nannizzia fulva
Microsporum nanum³	Pigs	Nannizzia obtusa
Microsporum cookei	Rare; man	Nannizzia cajetana
Microsporum vanbreuseghemi	Rare; man, animals	Nannizzia grubyia
Keratinomyces ajelloi	Rare; man, animals	Arthroderma uncinatum
Trichophyton terrestre	Rare; man	Arthroderma quadrifidum
Trichophyton mentagrophytes	Man, animals	Arthroderma benhameu

The Common Dermatophytes

			SPECIES	DISEASE IN MAN	GEOGRAPHICAL DISTRIBUTION
			Microsporum audouini	Prepuberal ringworm of the scalp; suppuration rare	Commonest in Europe, producing about 90 per cent of infections; in U.S. 50 per cent
	Small spore varieties		Microsporum canis	Prepuberal ringworm of scalp and glabrous skin; suppuration not infrequent; kerion occasional; from pets	Uncommon in Europe; responsible for about half the infections in U.S.
			Micropsorum gypseum	Ringworm of the scalp and gla- brous skin; suppuration and kerion common; from soil	Relatively rare in U.S.; common in South America
			Microsporum fulvum	Ringworm similar to that of M . $gypseum$	Same as above
			Microsporum ferrugeneum	Similar to M. audouini	Africa, India, China, Japan
and nair tourcies			Trichophyton tonsurans	Black-dot ringworm of scalp and smooth skin; sycosis; ony- chomycosis; suppuration com- mon; the hair follicles are atrophied	Common in Europe, Russia Poland, Italy, Near East, bu uncommon in U.S. until re cently
Invading the hair and ha		Endothrix type	Trichophyton violaceum	Black-dot endothrix in both scalp and smooth skin; onychomyco- sis; suppuration is the rule and kerion frequent	Common in Europe and Far East rare in U.S.
nding in	les	Endo	Trichophyton soudanense	Inflammation, scarring, ringworm of scalp	Central and West Africa
Inva	e variei		Trichophyton gourvilii		
000	Large spore varieties		Trichophyton yaoundi		
c I	E.J.	type	Trichophyton mentagrophytes	Commonest cause of intertri- ginous dermatophytosis of the foot ("athlete's foot"); ring- worm of smooth skin; suppura- tive folliculitis in scalp and beard	Ubiquitous
		Ectothrix type	Trichophyton verrucosum	Ringworm of scalp and smooth skin; suppurative folliculitis in scalp and beard; from cattle	Ubiquitous
			Trichophyton megnini	Sycosis is the most common lesion; infection of smooth skin and nails	Sporadic distribution; Sardinia Portugal
;	No Seroce	in hair	Trichophyton schoenleinii	Favus in both scalp and smooth skin; scutulum and kerion	Europe, Far East; rare in U.S.
Not invading the hair and hair follicles			Epidermophyton floccosum	Cause of classic eczema mar- ginatum of crural region; causes minority of cases of intertri- ginous dermatophytosis of foot; not known to infect hair and hair follicles	Ubiquitous, but more common i tropics
			Trichophyton rubrum	Psoriasis-like lesions of smooth skin; onychomycosis; mild suppurative folliculitis in beard	Common in Far East, tropic Europe, U.S.
N _{ot}			Trichophyton concentricum	Commonest cause of tinea im- bricata; infection of hair and nails uncertain	Common in South Pacific islands Far East, India, Ceylon; re- ported in South America; does not occur in U.S.

by continued culture on artificial media. In consequence many stock cultures of dermatophytes are atypical to a greater or lesser extent. Such variation is especially prone to occur on maltose "proof" agar, and Sabouraud recommends a milieu de conservation, the same medium but lacking sugar, which does not bring out the differential characters of all species; growth on this medium is much slower, but it has the virtue of postponing pleomorphic changes. Differential characteristics reappear on transfer to the maltose milieu d'epreuve.

Differential characteristics. Very many different kinds of dermatophytes have been described, some estimate as many as 200 or more, but many "new" species have been inadequately studied and often represent minor variants of established species. Primary differentiation into the three imperfect genera. Trichophyton, Epidermophyton, and Microsporum, is morphological and based on colonial morphology and the character of the macroconidium. A number of physiological studies have been carried out,81,181,219 and it has been found that certain species or strains are distinguished by requirements for vitamins or amino acids. These might provide a basis for identification, but so far at least, physiological characteristics have not been as useful for the differentiation of these forms as they often are for the characterization of bacteria.

Those characteristics established by the work of Georg⁸¹ and found to be useful are:

SPECIES REQUIREMENT

T. verrucosum	Inositol and usually thiamine
T. tonsurans	Thiamine
T. megninii	Histidine
T. equinum .	Nicotinic acid
T. violaceum	Thiamine

The test is carried out by planting the organism on deficient mediums.

While growing on the skin and its appendages, the thallus is differentiated only into hyphae and arthrospores; the latter, arising by fragmentation of the mycelium are to be regarded as oidia, as indicated earlier, though usually termed "spores" by dermatologists. The variety of differentiated structures, including aleuriospores (microconidia), and spindle spores or fuseaux (macroconidia), and vegetative structures, such as spirals, pectinate bodies, nodular organs, and racquet mycelium, appear on cultivation on artificial mediums. The differences are no doubt a consequence

of the nutritional state of the substrate and/or the semiparasitic existence on the host.

The first two genera listed above (the third does not invade hair) differ with respect to size and arrangement of the spores formed in the hairs. The genus Microsporum includes the small spore type (3 to 4 μ in diameter) and the genus Trichopyton, the large spore type (7 to 8 μ in diameter). This distinction is not absolute, for the very common species, Trichophyton mentagrophytes, forms small spores. Furthermore, the spores differ in arrangement. While the mycelium of Microsporum grows within the hairs, spores are formed only outside the hair and occur in irregular clusters in a kind of mosaic arrangement. The spores of Trichophyton, on the other hand, occur in chains inside or outside the hair.

Further differentiation of the organisms is made on the basis of location of the arthrospores with respect to the hair. The endothrix type (sometimes called black-dot ringworm) grows within the hair, and mycelium and chains of spores are found there. The hair often breaks off at the scalp line and gives an area of alopecia (usually cuboidal and accompanied by a kerion) and black dots of hair stubble. The ectothrix type forms spores only on the surface of the hair, though the mycelium grows inside. A third type is found in favus (T. schoenleinii) in which the organism grows in the hair but produces no

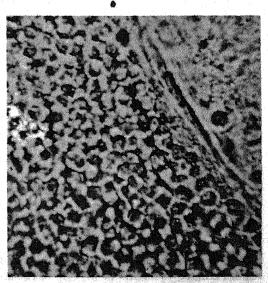


Figure 192. Endothrix tinea capitis (T, violaceum). Chains of spores within hair shaft, \times 100.

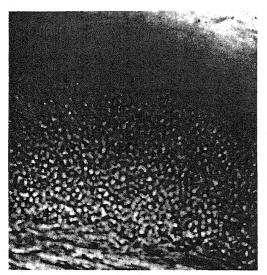


Figure 193. Ectothrix tinea capitis (*Microsporum audouinii*). Note the mycelium in hair shaft (lower left) with mosaic arrangement of arthrospores around hair shaft. × 400.

spores, only "air bubbles" in the hair shaft. The hair does not break off, but instead loses its color and luster and appears as a patch of gray hair.

It will be clear that differentiation of these genera may be approximated by, first, the clinical character of the disease and, second, by direct microscopic examination of epilated infected hairs, but identification is possible only by culture. In general, Trichophyton infections show a characteristic tendency to produce an inflammatory reaction with deep infiltration of the skin that is not usually produced by Microsporum infections; this difference is of some value in distinguishing between Microsporum infections and infections of the scalp with small-spore ectothrix Trichophyton. The animal strains of Microsporum such as Microsporum canis, however, also elicit inflammatory reactions. A severe inflammatory reaction with a raised mass of tissue, usually suppurating at many points, is called a kerion. Excessive fibrous scar tissue following an infection is called keloid formation.

Epidermophyton invades the superficial layers of the skin in tinea corporus and, in scales of the epidermis taken from the periphery of the lesion, the fungi are found as articulated filaments of mycelium breaking up into chains of round to oval arthrospores.

Pathogenicity. At present, the dermatophytes are known as parasites; they were once assumed to be obligate parasites either of man alone or of man and animals. The majority grow readily on laboratory mediums and also grow on such substrates as cereal grains, shed hair, horn debris, and sterilized fragments of straw in moist tubes and will remain viable in litter containing such materials for two to three years. If protected from dryness they may live on the wooden floors of shower rooms, dressing rooms, and dressing cabins, on mats, etc., for a considerable time. Furthermore. various dermatophytes have been isolated from soil and air,2 and it is probable that dermatophytes live a saprophytic existence, formerly unrecognized.

The dermatophytoses show, in many instances, a pronounced age and sex distribution, and there is some difference in the geographical distribution of the various species. Common ringworm or "gray patch" of *Microsporum audouini* of the scalp is confined to the young, occurring more often in boys than in girls, and it is rare after puberty. Others, such as the Epidermophyton species, occur for the most part in adult males. The distribution of intertriginous dermatophytosis of the feet, commonly known as "athlete's foot," in the young adult male is probably in large part an expression of risk. *M. canis* is more

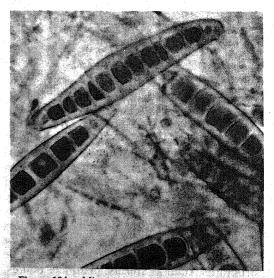


Figure 194. Microsporum canis macroconidia. The colony is fluffy white with a chrome-yellow reverse. Both micro- and macroconidia are found. Lactophenol-cotton blue; × 400.

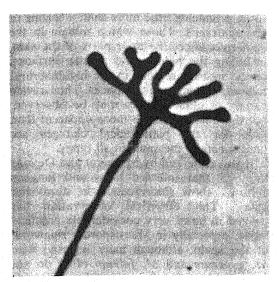


Figure 195. "Favic chandeier" of *Trichophyton schoenleinii*. Spores are absent in this fungus, but the peculiar mycelial arrangements are diagnostic. Lactophenol-cotton blue; × 400.

common in this country than in Europe. except England, and the reverse is true of *T. schoenleinii*, while *T. concentricum* is well known in the tropics and certain parts of the Far East but is rare in temperate climates.

Some dermatophyte species appear to be so closely adapted to man that they are unable to infect lower animals; human infection is transmitted by contact.53, 151 These organisms are termed anthropophilic dermatophytes. Others not only produce infections in experimental animals, but animals such as the cat and dog are natural hosts, and human infection may be acquired from them. The reservoir of animaltype dermatophytes is of considerable epidemiological importance;78, 138, 158 survey showed, for example, that 37 per cent of cats and 22 per cent of dogs examined at random in this country were infected. Organisms such as Microsporum nanum⁶ are commonly found infecting pigs but not man. All these are referred to as zoophilic dermatophytes. Some are regularly contracted from the soil, such as Microsporum gypseum, and are termed geophilic.63,68

There is also a high degree of specificity as to the tissues attacked. While, as indicated earlier, these fungi are well adapted to parasitize the horny layer of the epidermis, they appear to be unable to invade and infect other organs of the body. The intravenous injection of Microsporum spores or emulsions of virulent *T. mentagrophytes* does not produce an infection of the internal organs of susceptible animals; rather, the microorganisms introduced tend to become localized in the skin and to develop where it is damaged as by scarification. However, it has been shown that dermatophytes, as well as several saprophytes, can be "trained" to assume a yeast-like phase similar to the deep-infecting fungi. In this transient condition the organisms can invade deep tissues of experimental animals.¹⁸⁴

Growth of the fungus in the skin and hair is more or less equal in all directions, and the lesions produced tend to have a circular form. For this reason the Greeks named the disease herpes, a term which still persists though modified as herpes tonsurans, herpes circinatus, or herpes desquamans to distinguish the dermatophytoses from herpetic infection of virus etiology. The Romans associated the lesions with lice and named the condition tinea, meaning any small insect larva. This name is likewise in common use. The English ringworm is, of course, a combination of the Greek and Roman terms.

The clinical conditions produced are termed: tinea pedis (athlete's foot); tinea corporis (ringworm of body); tinea capitis (ringworm of the scalp); tinea cruris (ringworm of the groin or "jock itch," also commonly caused by Candida); tinea ungium

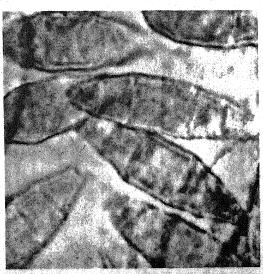


Figure 196. Microsporum gypseum (Nannizzia incurvata) macroconidia from the cinnamon-colored, powdery colony. Lactophenol-cotton blue; × 400.



Figure 197. Trichophyton mentagrophytes. Globose microconidia "en grappe" and spiral mycelium identify this fungus. The pencil-shaped macroconidia are identical to those of T. rubrum.

(ringworm of the nail or onychomycosis and commonly a Candida infection); and tinea imbricata (a special concentric ring form caused by Trichophyton concentricum).

With any species of dermatophyte, infection begins in the horny layer of the epidermis. Those which infect the hair follicles. hair, and nails soon invade these structures.

often producing little more than a scaling of the epidermis. Those which remain in the epidermis affect the drier parts of the skin. including the palmar and plantar surfaces, or the moister regions in the inguinocrural fold and the interdigital spaces. Thus a wide variety of clinical types may be observed. but the differences are more apparent than real, for the pathological changes are fundamentally the same in all types.

It was observed by Margarot and Devoze in 1925 that infected hairs and fungous cultures show fluorescence in ultraviolet This empirical observation has proved to be of very considerable practical value, especially in Microsporum ringworm of the scalp, although hairs infected with M. gypseum and M. fulvum often do not have this property. It is generally agreed that all hairs infected with Microsporum show a brilliant greenish fluorescence and those infected with T. schoenleinii a greenish but less brilliant fluorescence, both distinct from the bluish tint of normal skin. There appears to be some disagreement with respect to Trichophyton infection; it is reported by Davidson and Gregory³⁸ that in infections

^{*&}quot;Black light," commonly known as Wood's light because the radiation is filtered through Wood's nickel oxide glass, which holds back almost all the visible rays but passes the longer ultraviolet rays (peak at 3560 Å).

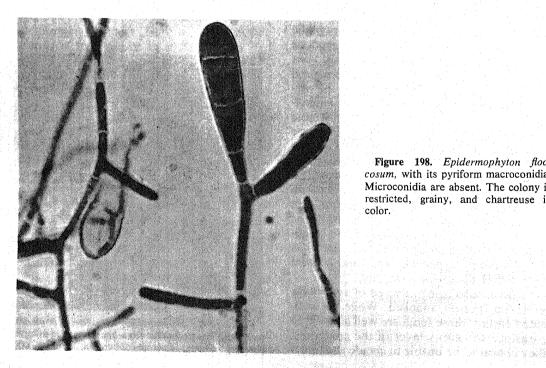


Figure 198. Epidermophyton floccosum, with its pyriform macroconidia. Microconidia are absent. The colony is restricted, grainy, and chartreuse in

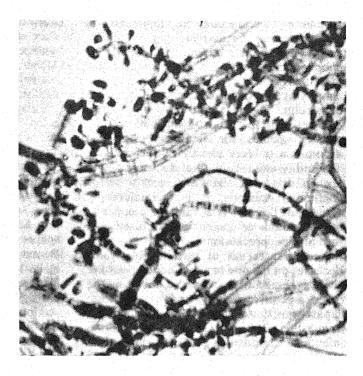


Figure 199. An unusual highly sporulating *Trichophyton rubrum*. Note the "teardrop" microconidia and elongate trichophyton type of macroconidia. The white fluffy colony has a wine-red reverse.

with some species the hairs fluoresce but in others they do not, while Lewis and Hopper¹⁴¹ state that all endothrix Trichophyton species show a dull bluish fluorescence of the infected hair, though hair infected with ectothrix Trichophyton does not fluoresce. It has been found that the fluorescence is due to the presence of a substance in the hair which can be extracted either with warm water after preliminary ether extraction or with dilute alkali. ¹⁸⁷ It is apparently not produced in culture, though some cultures (*T. tonsurans*) show some degree of fluorescence.

Immunity. Though an acquired immunity to infection has been demonstrated in experimental animals by a number of workers, the status of an effective immunity in man is uncertain. There have been some enthusiastic reports of the efficacy of vaccine therapy which are, perhaps, open to question. Much of the work that centers about local immunity appears to be uncritical; i.e., in many cases remarkable results have been reported but have not been regarded by immunologists generally as contributing materially to a solution of the general problem of local immunity. Hypersensitivity, however, is a common, though not invariable, manifestation of the immune response to dermatophyte infection, and desensitization procedures appear to have definite therapeutic value in certain cases. Normal serum appears to have some inhibitory effect on dermatophytes. 145

Hypersensitivity is manifested in two ways. One of these is the appearance of secondary, nonparasitic lesions on parts of the body remote from the infection. These are termed "ids," in a general sense mycid or dermatophytid, and more specifically microsporid, trichophytid, and epidermophytid. A similar condition, candidid, may result from a Candida infection. The mycid takes the form of a symmetrical eruption over relatively large areas, usually of the trunk, as a rash. The eruption may be vesicular with sterile content, papular, or lichenoid and is sometimes localized at the follicular pores. A rather frequent occurrence is a sterile vesicular eruption on the hands secondary to infection of the feet. It is generally believed that bits of mycelial debris from the lesion enter the blood stream and are eventually deposited in the skin, where they induce the local allergic response with destruction of the fungous elements. This view is based in part on a number of successful isolations of these fungi from the blood stream during the development of mycids. It is not known what part, if any, soluble substances liberated by the dissolution of the fungi play in the phenomenon.

Hypersensitivity may also be demon-

strated by the injection or application of preparations of dermatophyte cultures analogous to tuberculin and called trichophytin.36 The local and constitutional reactions that may be produced with trichophytin are much the same as those to tuberculin. The test does not differentiate between Microsporum and Trichophyton or their species, for all appear to contain a common or very closely related antigen. The utility and reliability of the trichophytin test in diagnosis form a subject arousing conflicting opinions. It is maintained by some that the test is a valuable adjunct to other methods of diagnosis when used with the proper precautions; others, however, regard the test as of questionable value because, on the one hand, infection does not always result in sensitization and, on the other, a positive reaction may only indicate a past infection unrelated to the condition in question. Persons with chronic T. rubrum infections frequently show an immediate wheal in response to trichophytin injection and essentially no delayed response. The relation of this to the chronicity of their disease is unknown but is interesting to speculate upon.

Laboratory diagnosis. As indicated earlier, classification of the dermatophytes is based largely on the clinical character of the infection, on colonial morphology on Sabouraud agar, and on minor difference in spore type. The differentiated structures developed in culture are, in some instances, of ancillary value. Fermentation reactions. emphasized by Castellani, are generally regarded as of little or no differential value. Animal inoculation is of minor importance unless the lesion is atypical and it is desirable to establish pathogenicity. Requirements for vitamins and amino acids and sexual mating are the only new tools to help in distinguishing species. These are of value in only a few cases. Laboratory diagnosis, then, is still a matter of looking at gross and microscopic morphology, an exercise in contemplative observation.

The specimen material must be chosen with some care and taken in abundance for both microscopic examination and culture. In ringworm of the scalp, epilated stumps of hairs may be taken and, in addition, scutula should be taken in favus and the contents of abscesses when the infection is suppurative. In infections of the smooth skin, scrapings from the scaly types should be taken

from the margins, rolling toward the normal skin, and the tops of vesicles may be clipped with small scissors in the vesicular types. Macerated epithelium may be taken from intertriginous infections, and nail scrapings and subungual hyperkeratotic masses from onychomycosis; the fungous elements are often more difficult to demonstrate microscopically in such specimens.

For direct microscopic examination the material is placed on a slide, a few drops of strong (20 to 40 per cent) potassium hydroxide solution added, the specimen covered with a coverglass, and the preparation heated slightly. Mycelial elements and spores may be found in such specimens. Permanent stained preparations may be made by mounting in Amann's medium, a lactic acid-glycerol-phenol solution containing cotton blue. As indicated earlier, sections may be stained by Gram's method or with methyl green-pyronine.

Sabouraud's agar containing cycloheximide (Actidione) 0.5 mg./cc. and Aureomycin 0.1 mg./cc. is the medium of choice for primary isolation as well as for determination of colonial character. A suitable substitute for Aureomycin is chloramphenicol or a penicillin and streptomycin combination. Inoculation should be relatively heavy and a number of plates prepared. Growth is relatively slow, usually 10 days to three weeks are required, and the dermatophytes grow well at 30° C.

THE PATHOGENIC YEASTS

The term, yeast or yeast-like, is the vernacular for a unicellular, nucleated organism which reproduces by budding. Such a definition is generally recognized as inadequate, in part because some yeasts reproduce by fission, in part because many produce mycelium or pseudomycelium under appropriate conditions, and in part because other fungi may exist in a unicellular yeastlike form which reproduces by budding, viz., the oidia described in the previous section. On the basis of sexual spore formation, some yeasts are ascomycetes, others are probably basidiomycetes (the ballistosporangenous yeasts), and still others have not been shown to have a sexual stage and are grouped with the Fungi Imperfecti. Clearly, then the term "yeast" is of somewhat uncertain significance; as commonly used, it refers to those organisms which exist usually or predominantly in a yeast-like form.

The yeasts belong in three fungal classes: the basidiospore-forming yeasts or Sporobolomycetaceae in the class Basidiomycetes, the ascospore-forming yeasts Endomycetaceae in the class Ascomycetes, and the asporogenous yeasts Cryptococcaceae in the Fungi Imperfecti. This last group contains the human pathogens. The industrial yeasts are, perhaps, the most familiar organisms. Saccharomyces cerevisiae, a member of the second group, is the common brewing yeast and occurs as two types: top yeasts are, perhaps the most familiar or of carbon dioxide and are found in the froth on the surface of the fermenting mixture, and the bottom yeasts, which sink to the bottom. Bread yeasts are usually top strains of S. cerevisiae. Another species of this genus. S. ellipsoideus, is the common wine yeast, occurring naturally on grapes and in the soil of vineyards, and its varieties are names for the various types of wine which they produce. These organisms are "perfect" yeasts, the cell body becoming an ascus during sexual union. Still other yeasts are lactosefermenters and are associated with the preparation of fermented milk beverages, such as kefir and koumiss, especially in southeastern Europe. Perhaps the commonest yeasts encountered as contamination in bacterial cultures and found growing on foods are the asporogenous *Rhodotorulae*; the pink or coral pigmented forms often observed are *Rhodotorula flava* or *R. glutinis*.

In view of the ubiquitous distribution of yeasts, not only in air, dust, and soil, but on the surface of the body and in the mouth, intestinal tract, and vagina, it is not surprising that these forms have been found in a variety of pathological processes. A great number of species have been described, most of them inadequately, in this connection. In many instances the yeast probably had no etiological relation to the disease, and in others the same yeast has been repeatedly described as a new species, thus giving rise to several synonymous names, and a very long list of "pathogenic" yeasts has accumulated.

Critical examination and consideration has now made it clear that only a very few species of yeasts are actually pathogenic for man and animals. The yeasts of medical importance produce a wide variety of diseases; these are listed in the accompanying outline with their basic differentiation and the classification generally accepted.

Classification of the Pathogenic Yeasts

Form-class: Deuteromycetes (Fungi Imperfecti) Form order: Pseudosaccharomycetales

Form-Family: Cryptococcaceae

Genus 1. Cryptococcus

Unicellular budding cells only, reproduce by blastospores pinched off the mother cell. Most are urease-positive. Cells surrounded by a capsule and starch-like substance produced. No carotenoid pigment.

Example: Cryptococcus neoformans (cryptococcal meningitis)

Genus 2. Torulopsis

Same as genus 1, but no capsule or starch-like polysaccharide produced.

Example: Torulopsis glabraeta (Torulopsis meningitis)

Genus 3. Pityrosporum

Mostly unicellular budding cells. Reproduction by blastospores that cut off from the mother cell by development of a cross-wall. Cell may adhere, forming short hyphal strands.

Example: Pityrosporum orbiculare (tinea versicolor)

Genus 4. Rhodotorula

Unicellular budding forms that may be encapsulated or produce pseudomycelium. Carotenoid pigments present.

Example: Rhodotorula mucilaginosa (rare pulmonary and systemic infections)

Genus 5. Candida

Reproduction by pinched blastospores; may form pseudomycelium or true mycelium. Ureasenegative usually. No capsules or carotenoid pigment.

Example: Candida albicans (candidiasis)

Genus 6. Trichosporon

Reproduction by blastospores and arthrospores. Mycelium and pseudomycelium formed.

Example: Trichosporon cutaneum (white piedra)

Genus 7. Geotrichum

Reproduction by arthrospore only. Forms a true mycelium. Some authors group this with Coccidioides immitis in the Arthrosporeae of Moniliaceae.

Example: Geotrichum candidum (rare pulmonary geotrichosis)

Candidiasis will be discussed in this section; the other pathogenic yeasts elsewhere.

CANDIDIASIS

"Monilia" is a nomen absurdum perpetrated in the nineteenth century. Unfortunately, the term is still used by clinicians and the public. Mycologically, the word Monilia refers to a black fungus growing on rotting wood, first described by Persoon. The correct term for the disease is candidiasis.

Candidiasis is one of the most frequently encountered of the fungous diseases. The organism Candida albicans, which is responsible for most infectious processes, is endogenous in man. It is part of the normal flora of the buccal cavity, large intestine, and probably the vagina. Under ordinary circumstances it is held in check by normal body defenses and other members of the normal flora. If this balance is changed as in debilitation of defenses, overdose of antibiotics, or local physiological change, the organism begins to proliferate at a rapid rate and establishes an infection. Not only is it ubiquitous, it is the most variable of of organisms in the clinical manifestations it may assume. Most commonly, it is associated with intertriginous dermatophytelike infection, as well as onychomycosis, tinea pedis, vaginitis, and thrush. It may also be involved in bronchitis, pneumonitis, and rarely meningitis and systemic involvement. 227

The organism displays a nutritionally governed type of dimorphism. Under favorable conditions of growth in the presence of fermentable carbohydrate, the organism grows as a budding yeast. 226, 234 In mediums without fermentable carbohydrate and with semi-anaerobic conditions and/or a high nitrogen content, the yeast elongates, forming pseudomycelium and mycelium accompanied by blastospore and chlamydospore production. When C. albicans is mixed with egg albumin or serum and incubated at 37° C., the yeast cells show "sprout" mycelium. This Reynolds-Braude phenomenon affords a rapid diagnostic procedure. 136 In tissue, elongation to form mycelium is commonly encountered. In experimental candidiasis, mycelium conversion occurred within two hours in the kidney after injection of C. albicans yeast. 120

The first description of a fungus of this

type occurring in disease was that of Langenbeck, who in 1839 found it in patches on the mucous membranes of the mouth and elsewhere at autopsy. Gruby (1842) confirmed this finding, and the organism was named *Oidium albicans* by Robin four years later. The generic name Candida has come to be commonly used, and the disease is termed candidiasis.

The causative organism. 236 In addition to the original Candida albicans, a considerable number of species of Candida have been described, including C. krusei, C. paropsilosis, C. tropicalis, and C. steallatoidea. The second most commonly encountered pathogen is C. tropicalis. It may be missed if the specimen is planted on a cycloheximide medium, as it is sensitive to this chemical. Together with C. albicans it is considered a pathogen. The other Candida are rarely isolated from disease processes. Some normal skin contaminants such as C. paropsilosis account for most Candida heart valve involvement. In fact, a large proportion of the current literature on these organisms is devoted to their differentiation, identification, and classification.²⁰⁶ Differentiation is made on the basis of various cultural characters, such as pellicle formation in liquid mediums, gelatin liquefaction, and the like, and differential fermentations. The last is the most practical laboratory method, but there is some disagreement as to the constancy of these characteristics. The R-B egg test described above is the most rapid method of identifying C. albicans.

These various strains are immunologically related and show marked cross-reactions. but the group is not homogeneous. A detailed antigenic analysis has shown the essential identity of C. albicans and C. tropicalis, and the separation of C. stellatoidea. Similar studies by Hasenclever and his associates 101, 102, 103 with C. albicans, for which chlamydospore production is the usual criterion for identification, showed that it is separable into two antigenic types, designated groups A and B. C. tropicalis falls into group A, C. steallatoidea into group B. Strains of C. tropicalis and C. steallatoidea, as well as C. albicans strains of both groups, are virulent for the mouse, 69 but the virulence of C. albicans for the rabbit appears to be significantly greater than that of the other species.

Pathogenicity. These fungi are commonly present in the mouth, vagina, and the intestinal tract in normal persons and

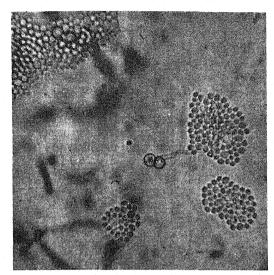


Figure 200. Live colony of Candida albicans which has produced a thick-walled chlamydospore on corn meal agar. (R. Murphy.)

are probably not highly virulent with respect to initiation of an infectious process. In fact, as in many of the mycoses, there is frequently a history of a debilitated condition or other predisposing factors. Several systemic conditions are known to favor Candida infection, principally diabetes mellitus. Others include vitamin deficiency (particularly of riboflavin and others of the B group), hypoparathyroidism, and lymphoblastomas, and overuse of broad-spectrum antibiotics. Candida infections have oc-

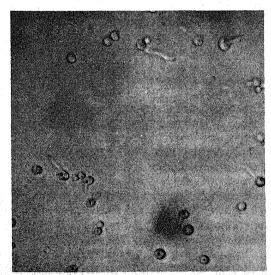


Figure 201. Sprout mycelium (R-B phenomenon) of *Candida albicans* after two hours in egg white at 37° C. × 400.

casionally heralded prediabetic diathesis and hypoparathyroidism before the disease was clinically apparent. In some instances in which they are associated with the development of a pathological process, there is some doubt as to their etiological role; in many others it is clearly evident that they are primary invaders and responsible etiological agents. Several workers, 134 particularly Louria and Brayton, 146 have demonstrated a humoral anti-Candida factor. Unlike an induced antibody, it remains at a particular level in normal individuals and is low or absent in diabetes, lymphoblastomas, and other diseases. Precipitating antibodies to soluble antigens of Candida are found in systemic disease.227

There is evidence that the treatment of other infections with the broad-spectrum antibiotics, especially the tetracyclines and chloramphenicol, increases the number of persons in whom Candida is found in the intestinal tract, vagina, and perianal infection, and it is related to the incidence of candidiasis which occurs as a sequel to prolonged therapy with these agents. While the suppression of susceptible microflora antagonistic to the fungi is probably concerned in the overgrowth of Candida, it has been suggested that such antibiotics may, in fact, enhance the virulence of these fungi or adversely affect the tissues concerned. There is a certain amount of experimental evidence supporting this. 20, 117, 118, 119 but its relative importance remains obscure.

Infections of the mucous membranes. Candidiasis of the mucous membranes is known as thrush and is one of the common mycoses. Intra-oral thrush, a superficial infection of the mucous membranes of the mouth, is the type most frequently observed and is considerably more common in nursing infants and children than in adults. In the past it has occurred in severe epidemic form in institutions such as foundling asvlums, but such epidemics are observed less often now. Thrush occurs more frequently in bottle-fed than in breast-fed babies, and the lesions not infrequently spread to the pharynx and even the esophagus. In adults, thrush generally occurs terminal to wasting diseases such as tuberculosis and cancer: dryness of the mouth associated with prolonged coma or near-coma appears to favor infection. It also occurs with some frequency as a mild vaginal infection in pregnant women which may or may not be associated with infection of the anus.

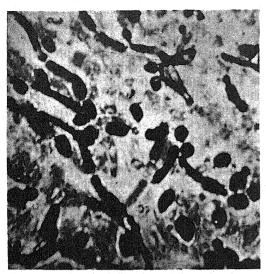


Figure 202. Candidiasis of kidney showing elongation of cells to form pseudomycelium and mycelium. Gram stain \times 600.

In the majority of cases the infection is mild and remains localized. Occasionally it may spread to other mucous membranes and to the skin, with the development of a generalized cutaneous eruption, intertriginous lesions, and Candida granuloma; such cases are sometimes fatal. In the cutaneous form in infants it is to be distinguished from infantile eczema and allergic hypersensitivities. The most common predisposing malady for cutaneous candidiasis is diabetes. Hematogenous spread with metastasis and abscess formation in the viscera has been observed.

The lesions appear as soft whitish patches, composed largely of fungous growth. They are readily removed and leave an eroded surface. There is some resemblance to a diphtheritic membrane, and lesions in the throat and on the tonsils have no doubt been mistaken for diphtheria. However, it is much easier to separate the membrane in candidiasis. On microscopic examination of the membranous material in wet preparation, the observation of a tangled mass of segmented mycelium admixed with budding yeast-like cells, desquamated epithelium, and leucocytes establishes the diagnosis. The fungus may be cultivated on Sabouraud's agar, but isolation in pure culture is more readily accomplished with cycloheximide agar. The isolated fungus must be differentiated from other yeasts.

Dermatocandidiasis. C. albicans is also causally associated with eczema-like lesions of the moist skin similar to those produced by E. floccosum. Infection of the folds between the fingers, erosio interdigitalis, is more common than dermatophytosis of this region, and is found most often in those whose hands are frequently wet. Perleche. an infection of the angles of the mouth, is another form of intertrigo produced by these fungi. Ill-fitting dentures may lead to local lesions under the plates. Candids, sterile vesicular or exudative lesions which appear on the hands secondary to a focus of infection elsewhere, are analogous to trichophytids and are a result of hypersensitivity.

The nails, chiefly of the fingers, are also attacked by C. albicans with the production of chronic paronychia. The condition is differentiated, clinically from onychomycosis by retention of the luster of the infected nail and the absence of vellowish discoloration, crumbling, and thickening of the soft tissues. The nail shows transverse ridges and eventually becomes thickened, distorted, and brownish in color, with frequent involvement of the surrounding nail bed (paronychia). Like interdigital infections, the infection is found most often in those whose hands are often wet.

On microscopic examination of nail and skin scrapings cleared in 10 per cent potassium hydroxide, hyphae may be present together with the budding yeast-like cells.

Bronchocandidiasis.31 There appears to be a distinct type of candidiasis of the respiratory tract which was first reported from Cevlon by Castellani. It has been observed in Europe and in this country but is apparently more common in the tropics; its frequency as a primary infection is uncertain. According to Castellani, two types of the disease occur: the mild form, chronic bronchitis characterized by dyspnea and cough, is afebrile, while the severe form is similar to tuberculosis and usually fatal.

The fungus may be demonstrated by direct microscopic examination and by culture, but such findings must be interpreted with caution,12 for the organism is frequently found in sputum in other diseases, especially tuberculosis. It is recommended that the throat be cleansed by gargling before the sputum is collected and that other contamination be avoided. Most strains are pathogenic for rabbits and mice, producing tubercle-like nodules within two or three weeks after inoculation.

THE SUBCUTANEOUS MYCOSES

Subcutaneous mycosis refers to a group of fungous diseases in which both the skin and subcutaneous tissue are involved but disseminiation to the internal organs does not occur or only rarely occurs. The etiological agents are classified among several unrelated genera. They have the following characteristics in common: (a) they are primarily soil saprophytes of very low grade virulence and invasive ability, and (b) in most human and animal infections they gain access as a result of traumatic implantation into the tissue. The list of organisms isolated from or designated as the cause of such conditions is long and varied.224 Bluegreen algae in monkeys and dogs, Protothecus zopfii (an algae) and celery blight (Cercospora apii) in man, and Beauvaria (a beetle fungus) in man and turtles are just a few of the interesting ones listed.84 This indicates that many, if not all, organisms have a potential to establish local infections under certain circumstances, depending on their adaptability and the response of the host.

Tissue response to these agents varies as to the etiological agent in question. In most cases the lesion tends to be localized, and the reactions that develop are similar to those elicited by a foreign body. Details of tissue response are discussed below.

The major disease types are: chromoblastomycosis, sporotrichosis, mycetoma (maduromycosis), and the recently described and relatively rare subcutaneous phycomycosis. Infection by agents of the three major groups is accompanied by a type of dimorphism. The organisms undergo a morphogenesis from their saprophytic form into a tissue or parasitic stage. In chromoblastomycosis and mycetoma the response seems to be to complex tissue factors and is called tissue dimorphism. Sporotrichum schenckii can be termed an example of thermal dimorphism. Mycetoma was discussed under the actinomycetes. The other organisms will be treated here.

CHROMOBLASTOMYCOSIS

Chromoblastomycosis was discovered by Pedroso in 1911, but his observations were not reported until 1920, the first case in the literature being reported from Boston by Medlar in 1915. The geographical distribution is wide, however, with the greatest number of cases being found in Puerto Rico and Brazil, and it is quite common in Costa Rica. Other cases have been observed in other parts of South America, Africa, and the Far East.²³ Present knowledge of the disease has been summarized by Carrión.²⁶

The causative fungus is closely related to the Hormodendrum-Cladosporium group of the Dematiaceae (Fungi Imperfecti) and was named Hormodendrum pedrosoi by Brumpt. Three types of sporulation are observed, the predominant type differing from one strain to another, and this has led to the splitting of strains into several genera with considerable confusion resulting. The new genus Fonsecaea was created by Negroni in 1936 and has gained some general acceptance, and the organism is, therefore, also commonly known as Fonsecaea pedrosoi. Since the Hormodendrum type of sporulation is not an outstanding characteristic. Carrión has proposed that the name F. pedrosoi be accepted as the only species and that four varieties, typicus, cladosporioides, phialophorica, and communis, be recognized on the basis of predominant type of sporulation. Presently four species are accepted.

ORE	TYPE	SPECI

- a. Cladosporium
 1. Phialophora verrucosa (c only)
 2. Cladosporium carrionii
 - 2. Cladosporium carrionii (a only)
- b. Acrothecia

 3. Fonsecuea pedrosoi (a, b, c). Spore is more elongate than compact; a, b and c sporulation is rare.
- c. Phialophora
 4. Fonsecaea compacta (a, b, c). Spores in chain are round and closely packed.

The (a) cladosporium type of sporulation is characterized by chains of acropetelously budding spores resembling Penicillium, the (b) acrothecia spore type has single conidia around the septa of the fertile mycelium, and the (c) phialophora type has a group of spores emerging from a phialid.

The organisms all have in common very slow growth of a gray to black-brown colony, lack of gelatin liquefaction, and identical tissue phase morphology. Mycelium is not formed in the tissues, and the parasitic phase of the fungus is a brown sclerotic cell which divides by central septation of the yeast-like cell. There is no budding. The granule, which is always brown, is easily seen in unstained material or in H and E stained tissue sections.

The disease is an infectious granuloma.

of the skin and subcutaneous tissues, occurring usually, though not always, on the feet and legs. It ordinarily begins with a small warty growth on the foot and extends upward through the development of satellite lesions. It almost always remains localized, metastases are very rare, and there are no constitutional symptoms. The disease develops very slowly; usually the case is of 10 to 15 years' duration at the time of examination, and some are known to have persisted for as long as 40 years. In advanced cases there is some elephantiasis of the affected limb, and great numbers of lesions. These vary somewhat and are of four general types: hard, elevated, pigmented nodules; large, cauliflower-like, prominent tumors; moderately elevated, dull red, scaly patches; and discrete or verrucous hyperkeratotic growths. The lesions are readily traumatized, and the disease may be complicated by secondary bacterial infection and ulceration. The sclerotic cells appear as spherical brown bodies, perhaps 12 μ in diameter, with a thick cell wall and granular protoplasm, and often show internal septation. They may be observed in biopsy specimens either within giant cells or free in the tissues, and are demonstrable in the epithelial debris obtained by scraping the lesions. The fungus may be cultured from such scrapings or from infected tissue. Chemotherapy is not satisfactory, iodide in large doses giving local improvement but not cure; excision

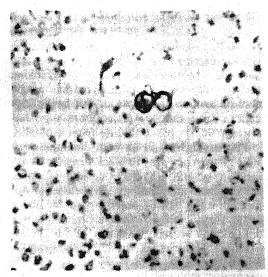


Figure 203. Chromoblastomycosis. Brown granule with rounded cells showing planate division. *Phialophora verrucosa*. Hematoxylin and eosin; × 400.

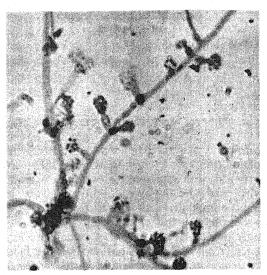


Figure 204. Phialophora verrucosa showing phialids and spores. Lactophenol-cotton blue; × 400.

of the early papillomatous lesion is recommended. The use of amphotericin B and other agents has been reviewed by Procknow and Loosli.¹⁷⁵

There appears to be no evidence of spread of the infection from one person to another. Since the disease tends to occur on the legs and feet of barefoot outdoor laborers in the tropics, it seems probable that the fungi are saprophytes normally present in soil or decomposing organic matter (they have been isolated from lumber, where they cause "blueing"). When introduced into the skin by a splinter or thorn or through some minor abrasion, they may at times assume pathogenicity. There appears to be good evidence at present for lymphatic and hematogenous spread of the disease in rare cases. Involvement of the conjunctiva and central nervous system has been recorded, although there is some confusion in reported cases with cladosporiosis. F. pedrosoi has been isolated from the lungs in rare cases, and the possibility of a pulmonary portal of entry must be considered. Bacquero and others have been successful in preparing a skin test antigen which causes a reaction in infected patients.

SPOROTRICHOSIS (Sporotrichum schenckii)

Sporotrichosis was first recognized by Schenck in this country in 1898 and a few years later in France by Beurmann and Ramond. It has since been found all over

the world, though the majority of reported cases are from the United States, especially from the Mississippii and Missouri Valleys, and from Mexico and France. It occurs as an occupational disease in pottery workers in Cuba, and the source of infection is presumably the packing material.⁸⁸ The organism is favored by moderate temperature and high relative humidity. Epidemics have occurred due to the growth of the organism on timbers in mine shafts.

Fungi of the genus Sporotrichum are characterized by the production of pearshaped conidia directly from the mycelium which arise both laterally and at the tips of delicate sterigmata on a conidiophore. They are described as "palm tree-like." The characteristic arrangement is not apparent in smears from cultures, and the spores are usually found free, but the arrangement is readily demonstrable in slide cultures. The hyphae are considerably more slender. 2 μ in diameter, than those of most molds. The character of the growth on agar is different from that of other molds; the colony is first soft and creamy in consistency, becoming more firm as the culture grows older, and there is no cottony mass of aerial mycelium. The growth also becomes darker with age, being at first a light tan which deepens to a dark brown or even almost black. When cultured on blood cystine agar at 37° C.. it grows as a budding yeast and is quite as "yeast-like" as Blastomyces or Histoplasma. Mycelium is not formed in the tissues, and the parasitic phase of the fungus is a cigarshaped body resembling an elongated yeast cell, 1 to 3 μ in breadth and 2 to 10 μ in length. These bodies are found within the leucocytes and apparently reproduce by budding; they are rarely observed in human infections. They are not spores. Occasionally, so-called asteroid bodies, consisting of a central fungous cell with deep-staining tissue material radiating from it, are seen in human tissue sections and material from experimentally infected rat testes. Because the organism is seldom seen in tissue, diagnosis is dependent on growing the organism. Surgical excision is contraindicated, as it tends to spread the infection.

The organism described by Schenck was named Sporotrichum schenckii. That isolated in France was supposed to be a different species and came to be known as S. beurmanni. The differences between the two are in pigmentation (S. schenckii being the lighter), the formation of fewer lateral spores

by S. schenckii, and the fermentation of sucrose but not lactose by S. beurmanni and the reverse by S. schenckii. There also appear to be some differences in the clinical type of disease produced. These differences are not constant, however, being subject to environmental modification, and it is apparent that the two are but a single species, S. schenckii. The name S. beurmanni continues to persist in the literature, however, giving the impression of two recognized species. The fungus infecting horses, S. equi, is also identical with S. schenckii.

Pathogenicity for man. The most common form of sporotrichosis in this country is cutaneous; the primary lesion, appearing at the site of some minor injury, often on a finger, fails to heal, ulcerates, and is followed by the appearance of a series of subcutaneous abscesses along the course of the regional lymphatics. The subcutaneous lymph vessels can often be traced as reddened lines. In the series of cases reported by Gonzales Bonavides, the disease occurred on the hand in 37.5 per cent, on the forearm in 25 per cent, on the arm in 6 per cent, and on the leg in 9 per cent, with lymphangitis extension in 37.5 per cent. Infection seldom extends beyond the regional lymph nodes, and cases of generalized hematogenous infection are rare in this country, though apparently more common in France. Metastatic lesions may appear in the liver or lungs and are most common in the testicles. The firm nodules in the skin are suggestive of syphilitic gummata, and probably some cases of sporotrichosis have been so diagnosed. Ocular sporotrichosis also occurs but is quite uncommon.89 Pulmonary involvement has occurred.203

Epidemiology. Direct transmission the infection from man to man has been observed but is very rare. A certain number of human infections have also been contracted, either directly through bites or indirectly by contact, from naturally infected horses and rats. In the great majority of cases, however, the fungus is introduced into the tissues from plants through some abrasion. Thus in 14 of a series of 18 cases the infection followed wounds from barberry thorns. The disease has been observed in florists and the fungus isolated from sphagnum moss.76 It has been observed growing free on grains, and Benham and Kesten¹⁷ demonstrated its ability to grow on barberry and carnations; in the latter a bud rot was produced, an interesting example of infec-

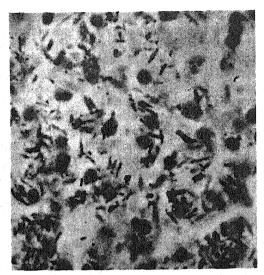


Figure 205. Sporotrichosis. Gram stain of heavily infected tissue showing elongate yeast-like cells.

tion of hosts as diverse as plants and man.¹¹⁵ It seems highly probable, therefore, that the fungus lives a saprophytic existence in nature, occasionally setting up an infection in man when mechanically introduced into the tissues.

Diagnosis. The diagnosis of sporotrichosis is established by demonstration of the fungus. The cigar-shaped parasitic cell is gram-positive, but may be found only rarely in gram-stained pus smears. These forms are relatively infrequent in human

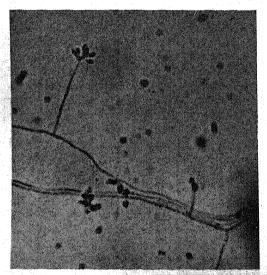


Figure 206. Sporotrichum schenckii mycelium with delicate conidiophore and conidia. Lactophenol-cotton blue; × 400.

material, and sporotrichosis is not excluded by failure to find them. The fungus is readily cultivated on Sabouraud's medium with cycloheximide from pus aspirated from unopened lesions. Rats are highly susceptible to infection, and inoculation of rat testes is of considerable diagnostic value. Injection of male rats intraperitoneally and intratesticularly with lesion material or culture results in a pronounced orchitis and generalized peritonitis with minute nodules on all peritoneal surfaces. The cigar-shaped cells are present in abundance in the rat lesion material and may be found readily in gram-stained smears.

Immunity. There is an immune response to infection manifested by the appearance of agglutinins (for spores), complement-fixing antibodies, and gel diffusion percipitins. They are of some diagnostic value, though the first two are somewhat nonspecific in that serums from persons with thrush or actinomycosis may give positive reactions. A cutaneous test with *sporotrichin*, a preparation analogous to tuberculin, is of similar specificity. A fluorescent antibody test has been used. 126

SUBCUTANEOUS PHYCOMYCOSIS

Since Lie-Kian-Joe and Emmons⁵¹ first described this entity in 1946, at least 100 more cases have been diagnosed24 and it may become a significant disease in tropical medicine. Unlike the other phycomycete diseases discussed in the following sections, there does not seem to be any underlying disease or predisposing factors apparent in the patients. The disease was first described in Indonesia but is now known in Uganda, Ghana, and India. In Africa it coincides with the geographic range of Burkitt's sarcoma; an insect vector has been postulated for both. The etiological agent was first designated Basidiobolus ranarum, a fungus associated with frogs and beetles but later shown by Greer⁹⁶ to be B. meristosporus. The disease is a long chronic process, often showing swelling of the arms, neck, chest, and trunk. The lesion involves the subcutaneous tissues and remains localized. Tissue section shows broad hyphae (20 μ) surrounded by an envelope of necrotizing eosinophilic debris. The lesion is a granuloma composed of chronic inflammatory infiltrate, foreign-body giant cells, fibroblasts, and thick-walled capillaries. Many

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of the cases seem to have healed spontaneously or were lost to follow-up. Nothing is yet established as far as serology or treatment are concerned. Another member of the Entomophthorales, the spider parasite Entomophthora coronata, has been isolated from nasal polyps in horses and man.²²

THE SYSTEMIC MYCOSES

The systemic mycoses involve any or all of the internal organs of the body as well as cutaneous, subcutaneous, and skeletal systems. The usual portal of entry for the etiological agents involved is the lung, in contrast to the other mycoses. 95 Infections are extremely common, especially in the United States, but the vast majority of cases are inapparent. In asymptomatic disease the diagnosis is most often made through a number of tests. Sensitization, which reflects present or previous experience with the organism, is detected by means of skin test or other immunological procedure. Its occurrence may be revealed by the presence of healed lesions observed in roentgenographic examination or at autopsy after death due to other causes. If the infection is symptomatic, the clinical signs may be those of a mild, self-limited disease, or the infection may become progressive with severe symptoms, tissue damage, and frequently death.

The organisms involved often show a predilection for a particular organ or type of tissue. Histoplasma capsulatum is an intracellular parasite of the reticulo-endothelial system; Cryptococcus neoformans prefers the central nervous system, Blastomyces dermatitidis usually involves cutaneous and mucocutaneous tissue. On the other hand, any one of the organisms can elicit the same type of symptomatology and tissue response and can mimic many other systemic or cutaneous diseases.

The diseases will be discussed in three sections: thermal and tissue dimorphic fungi, cryptococcosis and related fungi, and the rare mycoses.

THE DIMORPHIC SYSTEMIC FUNGI

One of the most remarkable and interesting phenomena exhibited by the fungi that infect man is dimorphism. The organisms involved exist in a saprophytic or mycelial phase in nature but undergo a morphogenesis to a parasitic stage during the process of infection. The change is apparently an adaptation to an unfavorable environment and to a degree is inherent in most all fungi. Several harmless saprophytes and dermato-

Thermal Dimorphic Fungi

DISEASE AND ORGANISM	saprophytic phase (25° C.)	parasitic phase (37° C.)
Histoplasmosis Histoplasma capsulatum	Septate mycelium, microconidia tuberculate chlamydospores. Colonies white-beige and fluffy.	Budding small yeast 1 to 5 μ Large yeast (5 to 12 μ) in African histoplasmosis H . duboisii
North American blastomycosis Blastomyces dermatitidis	Septate mycelium, microconidia pyriform, globose, or double. Colonies white-buff and fluffy or glabrous.	Thick refractile wall, budding yeast 8 to 20 μ . Broad-based bud
South American blastomycosis Blastomyces brasiliensis	Similar to B. dermatitidis.	Large, multiple, budding yeast cells 20 to 60 μ .
Sporotrichosis Sporotrichum schenckii	Pyriform conidia on delicate conidio- phores. Delicate septate hyphae. Colonies verrucous white with black underside.	Fusiform, oval, budding cells in culture 5 to 8 μ . Asteroid, cigar-shaped bodies only in tissue
Keloid blastomycosis Loboa loboi	Not known in culture.	Single, budding yeast, usually remain attached in long strands 8 μ . Exaggerated granulomatous and keloid response.

Tissue Dimorphic Fungi

DISEASE AND ORGANISM	SAPROPHYTIC PHASE	PARASITIC PHASE
Coccidioidomycosis Coccidioides immitis	Branched, vegetative mycelium which fragments into barrel-shaped arthrospores. Colonies moist and wooly, "moth eaten."	Spherules 10 to 80 μ with endospores.
Rhinosporidiosis Rhinosporidium seeberi	Not known in culture.	Spherules 100 to 300 μ with endospores.
Adiospiromycosis Emmonsia parva and var. crescens	Dense, dry, flatish, tan colony, aleuriospores on pedicits, 3 to 4 μ .	Spherules 40 μ (E. porva), 500 μ (var. crescens) also induced at high temperature (40° C). No endospores.

phytes have been induced into a transient yeast-like stage in which they are invasive for deep organs of experimental animals. 180, 184 The organisms well known as agents of systemic disease are generally unrelated species that seem to share an exaggerated ability to adapt to mammalian tissue. Infection, as far as the organism is concerned, is essentially a blind alley, as it is usually of no benefit in disseminating the species. The soil form produces the infectious spores, man-to-man transmission is essentially unknown, and the organism usually dies with its host.

The factors that have been cited as important in bringing about the transformation of the saprophytic to the parasitic stage are many. 167 Among others, combinations of temperature, carbon dioxide tension, reduced oxidation-reduction potentials, free sulfhydral groups, and liquid mediums have been described as necessary. 144, 190, 196 In several organisms transformation is produced in tissue or a simulated environment not necessarily related to temperature. The organisms seem to fall into three categories: the thermal dimorphic fungi, the tissue dimorphic fungi, and nutritional dimorphism (see section on Candida). In thermal dimorphic fungi, morphogenesis is to a budding yeast-like (Y) stage from an elargate saprophytic mycelial (M) phase. There may be ancillary factors necessary, as already mentioned, but yeast stage growth in vitro and in vivo is governed by temperature. This has been shown by injecting poikilothermic animals with the organisms (either growth phase). In animals incubated at 25° C. infection occurred with mycelium in the tissues; if they were incubated at 37° C. infection was accompanied by trans-

formation to yeast stage growth.197 It has been speculated that a temperature-sensitive enzymatic pathway is involved. In tissue dimorphic fungi, the associated parasitic stage is not a budding yeast but assumes a variety of forms, including a spherule in coccidioidomycosis and adiospiromycosis; a planate dividing round cell in chromoblastomycosis, and a distorted mycelial ball in mycetoma. Injection of frogs with Fonsecaea pedrosoi causes infection with associated granule formation at 25° C.¹¹¹ and, similarly, lizards and frogs infected with Coccidioides immitis produced spherules at room temperature.111 The thermal dimorphic fungi are a well-delineated group and are described in the first of the two accompanying tables. The tissue dimorphic fungi are less well defined and are described in the second table. The tissue dimorphic fungi also include the organisms of chromoblastomycosis and mycotic mycetoma previously discussed. Most of these diseases are treated with amphotericin B.50, 175, 222

NORTH AMERICAN BLASTOMYCOSIS

The fungous disease known as North American blastomycosis was first observed in Baltimore in 1894 by Gilchrist and is sometimes called Gilchrist's disease. The great majority of cases have been found in this country,216 concentrated in the north central and eastern states,202 hence the name American blastomycosis. The disease has been observed often in the state of Illinois and is occasionally called Chicago disease. At least five cases have been reported and substantiated from Africa, which considerably extends its endemic range.⁵² Analysis of presumptive and inadequately described cases is difficult because of the usual failure to distinguish this disease from European blastomycosis (cryptococcosis), or African histoplasmosis (large yeast *H. duboisii*). *B. dermatitidis* may also exist in a small yeast form, which is difficult to differentiate in tissue from the histoplasmosis organism.

The causative organism. This fungus was isolated in 1896 by Gilchrist and Stokes from a second case of the disease and named Blastomyces dermatitidis. It is dimorphic. occurring only as a unicellular, budding, yeast-like form in the tissues, but as a mycelial form in culture. The unicellular form may be observed in potassium hydroxide preparations of pus or sputum. The cells are large, 8 to 10 μ in diameter, round or oval, doubly contoured, and multinucleate. and they show a single broad-based bud. The granular content of the cells distinguishes them from air bubbles or fat droplets, and identification is practically certain if budding cells are found. The perfect stage has recently been described Ajellomyces dermatitidis and is in the family Gymnoascaceae. The organism is, therefore, closely related to the dermatophytes.

Colonial morphology. This unicellular morphology also occurs in cultures on blood agar, microscopic examination showing budding cells with a few rudimentary hyphae. The colony developing at 37° C. is wrinkled and waxy and somewhat similar in appearance to those of the tubercle bacillus. In culture on Sabouraud's agar at 25° C.

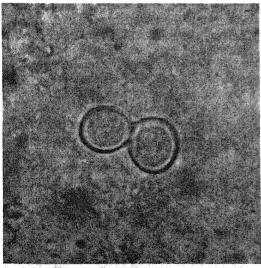


Figure 207. Potassium hydroxide preparation of pus showing yeast phase of *Blastomyces dermatitidis*. Note the broad-based bud and thick wall. × 400.

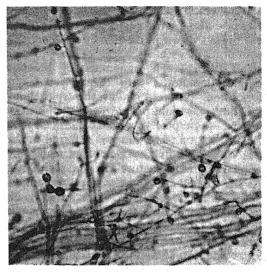


Figure 208. Mycelial phase of *Blastomyces dermatitidis*. Chrysosporum type of conidia. Lactophenolcotton blue: × 400.

the mycelial form appears. The colonial morphology is somewhat variable, and three types are distinguished. The "mealy" type is most often observed in primary isolation cultures and is similar to the growth on blood agar; microscopically the fungus is transitional between the unicellular and mycelial forms, with many of the cells tending to form articulated chains. After one or two transfers, the growth may assume a prickly surface, the prickles or spicules consisting of closely packed mycelial filaments which tend to become loose and cottony with continued incubation. The third type, sometimes observed on primary isolation and commonly found on laboratory cultures which are frequently transferred, is characterized by a white colony growth with abundant aerial mycelium; in these colonies the unicellular form has completely disappeared and growth consists of septate hyphae. Conidia are borne on lateral conidiophores. The mycelial form reverts to the unicellular type in experimental infections or when cultivated on practically any medium at 37° C. An odd metabolic product of its metabolism is ethylene.

Pathogenicity for man.²⁰¹ There are three types of disease processes: (a) primary cutaneous, (b) primary pulmonary, and (c) systemic, which may result from either of the former. Most infections are acquired through the pulmonary route. The primary skin lesion begins as a small firm papule

about which secondary nodules develop which enlarge with coalescence. The process breaks down in the center and becomes suppurative, with discharge of pus through small fistulas. The inflammatory reaction is granulomatous, with connective tissue formation, proliferation of the epithelium, mononuclear infiltration (pseudoepitheliomatous hyperplasia), and sometimes giant cell formation. The fungus is found in the pus of minute miliary abscesses which are a characteristic feature of the pathology. As the disease progresses, therefore, a large elevated mass of tissue, with an irregular ulcerated surface, is formed and oozes pus from multiple small openings upon pressure. The resemblance to epithelioma or tuberculous ulcer is sometimes striking. Lesions with pronounced epithelial proliferation simulate tuberculosis verrucosa. The process spreads slowly through the subcutaneous tissues and often becomes generalized by way of the blood stream. There is healing in the center of the lesions with scar formation. Biopsy for histological or cultural examination should be taken from the active edge of the lesion.

Primary infection of the lungs often closely resembles tuberculosis or histoplasmosis clinically, with cough, pain in the chest, weakness, sometimes hemoptysis, and productive sputum late in the disease. The pathological picture is variable in that there may be focal or diffuse consolidation, and the abscesses may be miliary or there may be larger nodules. Cavitation occurs but is limited to small areas. The microscopic picture also resembles tuberculosis, and sometimes it is difficult to differentiate unless the budding cells are found.

With generalization from primary foci of infection, multiple small abscesses of hematogenous origin occur throughout the body. They are most common in the subcutaneous tissues and differ from the primary skin lesions in that they develop without pain or marked erythema, are soft, and evacuate considerable quantities of pus when opened. In contrast to the primary skin lesions, which most commonly occur on exposed parts of the body, the secondary subcutaneous abscesses are usually found in covered areas. Secondary abscesses also commonly develop in the viscera, in muscles, under the periosteum, and especially in the prostate. The eye may rarely be affected.62 The disease may be chronic and persist for years, but in the generalized infection there is a septic febrile reaction and the case fatality rate is high. The diamidines, stilbamidine and 2-hydroxystilbamidine, have therapeutic activity¹⁷⁵ but relapses are not uncommon. More recently, amphotericin B has given very promising results.⁹⁹ Saramycin (X5079C) is now being tested.

The source of infection is not clear. Very often the primary skin lesion develops from a wound infection, and a large proportion of the cases have been in farmers. There is reason to believe that in most cases pulmonary infection is primary, presumably acquired by inhalation, and in some cases there are no other lesions, or cutaneous lesions may be overtly primary but secondary to the lung infection. Males are much more frequently attacked, and the incidence is higher in the 20- to 50-year-old group. The fungus has been recovered from soil⁴⁰ but very rarely in contrast to other pathogenic fungi. The yeast cells of the organism tend to lyse when placed in soil. The reason for this is as yet unknown. 154

Unlike other systemic mycoses, infection with *B. dermatitidis* is a progressive disease; spontaneous remission is uncommon.

Pathogenicity for lower animals. The inoculation of experimental animals is not uniformly successful and has no diagnostic utility. Mice are more susceptible than guinea pigs, and rabbits are almost completely resistant. Small caseous nodules develop on the peritoneal surfaces of intraperitoneally inoculated animals, and the type of tissue reaction varies with the resistance of the animal and the virulence of the strain of fungus from frank abscess formation to tubercle-like lesions. An instance of canine infection apparently transmitted to man has been reported.

Immunity. There appears to be little or no effective immunity in man to the spread of the infection since generalization is common. Complement-flxing antibodies are produced, however, and the titer is related to the severity of the infection. 64, 65 Long persisting titers are generally considered a sign of poor prognosis. Hypersensitivity to the cell substance of the fungus occurs also and is manifested as a delayed tuberculin-like response to intradermal inoculation of suspensions of killed cells. 152 Unfortunately, there is a cross-reaction with histoplasmosis

(or histoplasmin) and to a lesser extent coccidioidomycosis (or coccidioidin). The hypersensitivity is of practical importance in that it contraindicates the therapeutic use of iodides; desensitization may be accomplished with vaccine. It has not as yet been possible to demonstrate an effective immunity to the experimental infection in laboratory animals. Other serological procedures useful in diagnosis and prognosis are the gel diffusion test and fluorescent antibody procedure.

Diagnosis. As indicated above, the yeastlike unicellular form of the fungus can be demonstrated by direct microscopic examination of pus or sputum mounted in potassium hydroxide. Positive complement-fixation and skin tests are of value in pulmonary and systemic infections. An unequivocal diagnosis can be established, however, only by isolation and identification of B. dermatitidis. This is usually not difficult, as the fungus grows readily on cycloheximide bloodagar at room temperature. After growth has been established, it is necessary to confirm the diagnosis by showing the dimorphism to the yeast stage. This is done by taking mycelium from the cycloheximide bloodagar plate and planting on plain blood agar and incubating for several weeks at 37° C. The yeast stage of Blastomyces and Histoplasma are sensitive to cycloheximide. 153

COCCIDIOIDOMYCOSIS⁶¹

Coccidioidomycosis, coccidiomycosis, or coccidioidal granuloma was first observed in Argentina in 1892 by Posados and by Wernicke; two years later it was described independently in California by Rixford. The causative organism was thought to be a protozoan and named Coccidioides immitis. It resembled the coccidia group of protozoan parasites, a group that includes Eimeria coccidiosis of chickens and such other bird pathogens as Isospora. Ophuls and Moffitt showed by culture that it is a fungus, but this does not invalidate the name. Its relationship to other fungi is uncertain. Because it is more or less nonseptate in its early growth and there is resemblance of spherule production to sporangia formation, it was long considered an imperfect phycomycete. Some authors place it along with Geotrichum in the tribe Arthrosporeae of the Moniliaceae (Fungi Imperfecti).235 This evades the issue but will suffice until true relationships are discerned. Though originally reported from South America, the disease appears to be uncommon and is most prevalent in the San Joaquin Valley in California and in the dry regions of the southwestern United States and north central Mexico. There are scattered reports from Central America and South America. The organism is associated with a particular ecologic niche known as the Lower Sonoran life zone. Reports of rare cases in other parts of the world are probably the result of transmission by fomites. A coccidioides-like disease is recognized in Russia, the etiology of which is not yet well described.

The causative organism: parasitic stage. This fungus differs markedly from the yeastlike fungi in that it never reproduces by budding, and it differs from most other pathogenic fungi in that it reproduces within the tissues exclusively by a process of endogenous spore formation. The newly liberated spores are small mononucleate spheres 1 to 3 μ in diameter and appear as a central, deeply stained mass of protoplasm surrounded by a double-contoured cell wall. The cell enlarges, soon becoming multinucleate, and eventually reaches a diameter of 50 to 60 μ . A central vacuole appears early and in later stages occupies a large portion of the cell, the protoplasm appearing as a thin peripheral layer. The peripheral pro-

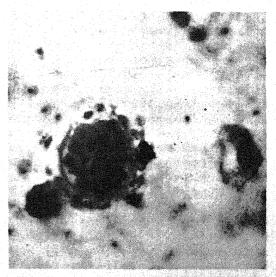


Figure 209. Breaking spherule of *Coccidioides* immitis in liver tissue with endospores. Gridley periodic acid-Schiff hematoxylin; \times 400.

toplasm becomes vacuolated by cleavage planes and an indefinite number of multinucleate protospores become delimited by the formation of cell walls; the protospores in turn subdivide to form spores and the entire cell assumes a function similar to that of a sporangium. With rupture of the cell wall the mature spores are liberated, and the developmental cycle begins again.137 It is at rupture time that neutrophils invade the area and the antigen released induces effective immunity. The effective antibody seems to be directed against newly released endospores. The same type of spherule-endospore cycle occurs in rhinosporidiosis. Spherules are also found in adiospiromycosis. All of these forms may be observed in the tissues and in pus, though recently disseminated spores are difficult to demonstrate. Hyphal forms have been observed in the tissues but are very rare. The morphological phase found in the tissues is determined by various environmental factors.33

The causative organism: saprophytic stage. The fungus will grow on a variety of sugar-containing mediums, but Sabouraud's agar is the medium of choice. It grows best at 37° C. under aerobic conditions. On artificial culture mediums the growth is that of a mold. The colony may be smooth and waxy when young, aerial mycelium is soon formed, and it becomes gray or brownish in color. Fragmentation with the formation of artho-

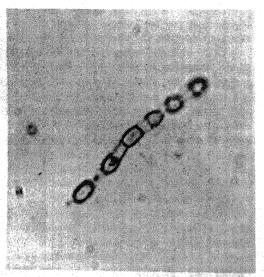


Figure 210. Mycelial phase of C. immitis. Barrelshaped arthrospores with empty cells in between. Lactophenol-cotton blue; × 400.

spores begins on side branches which are double-sized distally. The arthrospores are barrel-or cask-shaped, are thick-walled, and have an empty space between successive spores.

Pathogenicity for man.²¹⁴ For many years infection with C. immitis was known only as coccidioidal granuloma, a severe disease in which the case fatality rate was thought to be 90 per cent or more. Infection with this fungus is, however, much more prevalent and less severe than had been supposed. It is estimated that 10 million people in the southwestern United States have or have had coccidioidomycosis. Healed lesions of coccidioidal granuloma are found at autopsy of persons dying of other causes, and similar arrested lesions have been produced in white rats. Furthermore, it was shown by Dickson in 1937 that the disease may take a second form (known as "valley fever" or "desert rheumatism"), a benign, acute, self-limited respiratory infection, in addition to the chronic, progressive granulomatous disease. He suggested that these be designated primary and secondary or progressive coccidioidomycosis respectively, and these terms are now coming into general use. It is now felt that the experience of the benign self-limited disease confers a rather effective immunity. Coccidioidomycosis is the most virulent and most dangerous of all the fungous diseases. Many laboratory accidents have occurred. sometimes fatal. Though the dissemination rate is less than or near 1 per cent, it may be 10 times that in dark-skinned individuals. Any extension beyond the benign selflimited disease must be considered very dangerous for the patient and treated as such. In South America the infection is known as Posada's disease.

Primary coccidioidomycosis. The infection is acquired by inhalation of the spores, and the disease varies in severity in recognized cases from that of a common cold to that of cases resembling influenza with pneumonia, cavitation, and high fever. In a small proportion of the cases, possibly 5 per cent, an exanthem-like erythema nodosum or erythema multiforme occurs, and the disease is recognized as "the bumps," desert rheumatism, valley fever, San Joaquin fever, etc. Persons showing this allergic response very rarely develop serious progressive disease. Many infections are symptomless, however. In a group of 1351 cases

studied by Smith et al., 60 per cent were symptomless, and dissemination with development of the progressive disease occurred in about 1 per cent of the clinically apparent infections. They also noted that dissemination occurred 10 times as frequently in the Negro, Mexican, Portuguese, or Indian as in the white. 116 Minor "epidemics" may occur such as that described by Davis, Smith, and Smith³⁹ in which seven of 14 persons who spent two days on a desert field trip near the San Joaquin Valley became infected, and Goldstein and Louie reported that, of several thousand soliders exposed during military training in an endemic desert area, 75 contracted the disease. All but one made rapid progress to complete recovery, but the remaining individual developed a generalized infection and coccidioidal granuloma. A similar incident occurred in a prisoner-of-war camp near Florence, Arizona, and in a housing development called Paradise Valley near Phoenix. Arizona. The disease is also a major problem in northern Mexico. Spontaneous recovery is the rule, and only rarely does the disease progress to the secondary type. Residual pulmonary lesions, closely resembling healed tuberculous lesions, are common.¹⁰⁵ They differ from healed histoplasmomas or tuberculomas in that they rarely calcify.

Secondary or progressive coccidioidomycosis.211 The progressive type of infection results in cutaneous, subcutaneous, visceral, osseous, and central nervous system lesions, as well as extension of lesions into the lungs. If the disease is well established in the lung, it rarely remains exclusively pulmonary. The cutaneous type closely resembles blastomycosis but is a much more severe disease, with fever and a greater tendency to hematogenous spread. Elsewhere the lesions closely resemble those of tuberculosis, and, in fact, differentiation from that disease may often be possible only by demonstration of the fungus. Meningeal involvement is nearly always fatal. It is not known whether the progressive type of disease results from a new infection or reactivation of arrested primary lesions, probably the former. Amphotericin B is reported to be an effective chemotherapeutic agent in the disseminated infection, 32, 213 but a more effective drug is greatly needed. As in the treatment of all systemic fungous diseases, the toxicity, especially the nephrotoxicity, is a major side effect. Other side effects are fever, anemia, phlebitis, and various allergic responses.

Pathogenicity for lower animals. The disease occurs naturally in domestic animals including cattle. 150 sheep, and dogs and also in certain wild rodents in endemic areas. The latter include three species of pocket mice. Perognathus baileyi, P. penicillatus, and P. intermedius: the kangaroo rat, Dipodomys merriani; and the grasshopper mouse, Onychomys torridus. The disease can be disseminated to a new area from the carcass of an animal that has died from it. Experimental animals are readily infected; both rabbits and guinea pigs are susceptible to intraperitoneal inoculation and mice to intracerebral inoculation. Mice, inoculated intracerebrally 66, 127 or intraperitoneally, are used for virulence assay.

Immunity. Since it is now apparent that infection with *C. immitis* can take a mild form from which spontaneous recovery is the rule, that arrested lesions occur with some frequency, and that the case fatality rates in the severe progressive type of disease are not as high as once thought, it is indicated that there is, on the one hand, an appreciable natural resistance to infection and, on the other, an effective immune response.

The immune response is manifested in part as the development of a hypersensitivity to the parasite. Coccidioidin¹⁷⁷ is, like tuberculin (OT), prepared from liquid cultures of the parasite, but there is some difficulty in reproducing potencies. An immunologically active polysaccharide appears to make up at least a part of the active principle, producing both skin reactions and precipitin reaction. Coccidioidin is inoculated intradermally, a positive reaction appearing in 24 to 48 hours. The hypersensitivity appears in a few days to a few weeks after infection, recent infections and severe infections resulting in stronger reactions than old infections and mild infections respectively. The test appears to be highly specific, though there is as yet some disagreement as to its interpretation.

Humoral antibodies are formed also, and complement-fixation^{169, 212} and precipitin tests, using an autolysate of culture as the antigen, have been developed and are specific. In general, serum antibody is not detectable prior to the development of skin sensitivity. Precipitin appears first and is demonstrable in 50 per cent of cases by the

end of the first week of the disease and in 90 per cent or more by the third week, and then gradually falls until no titer is demonstrable after seven months. The complement-fixation reaction becomes positive more slowly, with about 8 per cent positive reactions by the end of the first week, and reversion becomes apparent by the second month. In general, then, precipitating antibody characterizes the primary stage of the disease with about 78 per cent positive reactions as compared with 55 per cent positive complementfixation reactions, while complement-fixing antibody occurs in the secondary or progressive stage in 98 per cent of cases in contrast with 36 per cent positive precipitin reactions. Thus the titer of complementfixing antibody has considerable prognostic significance; rising titer is indicative of the development of the progressive granulomatous form of the disease, often before this is evident clinically.212 Vaccines of various types have been developed and tested, but their efficacy is yet to be determined. Gel diffusion and fluorescent antibody techniques also have been developed. 125

Diagnosis. The laboratory diagnosis of coccidioidomycosis is dependent upon the demonstration of the parasite, the diagnostic value of a positive coccidioidin reaction being somewhat uncertain. As indicated earlier, it may be found on direct microscopic examination of pus, spinal fluid, and tissues but may be difficult to demonstrate in the sputum. Lactophenol-cotton blue or Mallory's eosin and methylene blue may be used for staining. Though C. immitis grows without difficulty on sugar-containing mediums, it has been cultured in a surprisingly small proportion of cases. It grows on cycloheximide medium. There is a very considerable hazard in culturing the fungus owing to the infectivity of the spores, and special precautions are required.⁷

Epidemiology. In the endemic areas of the southwestern United States, the proportion of reactors to the coccidioidin test is high, ranging from 46 to 90 per cent. The prevalence of infection in these regions appears, therefore, to be much higher than suspected earlier. A very large proportion of these reactors have no history of coccidioidomycosis.²¹⁴ It has been estimated that only 5 per cent of infected persons show sufficiently pronounced clinical symptoms to be detected and diagnosed.

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Though, as indicated earlier, the disease occurs in domestic animals and certain wild rodents, there is no evidence of direct transmission of the infection from animals to man, although dead animals may contaminate the soil in which they are buried. It seems likely that both man and animals are infected by the inhalation of spores contained in dust; there is frequently a history of exposure to dust storms in human cases, and the fungus has been isolated directly from dust. *C. immitis* is a saprophyte which infects animals and man by chance; the reservoir of infection appears to be in the soil.^{39, 43, 140}

RHINOSPORIDIOSIS

Rhinosporidium seeberi is the name given to the agent of a chronic granulomatous disease characterized by production of polyps or other manifestations of hyperplasia on mucous membrane surfaces. The disease is characterized by friable, highly vascular, sessile or pedunculated polyps which may appear on almost any mucosal surface. The nose, nasopharynx, or soft palate is most often involved. The conjunctiva and lacrimal sac are also commonly involved. Lesions

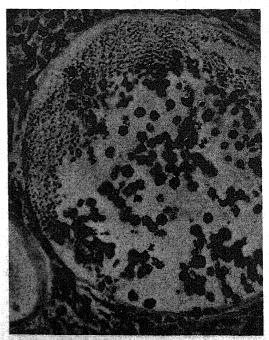


Figure 211. Giant spherule of Rhinosporidiosis in nasal polyp. Hematoxylin and eosin; × 400.

MANUFACTURE STATE

have been found on larynx, penis, vagina, rectum, and skin. The disease was first reported in 1896 by Seeber in South America. At least 200 cases are now known in Brazil. over 1000 in India and Ceylon; very rarely are cases reported in other parts of the world. Histologically, a typical tumor is composed of squamous or columnar epithelium showing a stroma of connective tissue, capillaries, and fungal spherules. The spherules are 10 to 200 μ in diameter and may contain 16,000 to 20,000 endospores. The cycle of development is similar to that in coccidioidomycosis. The organism has never been cultured. Some authors describe a protozoan-like karyosome along with a nucleus, and perhaps the disease, like Pneumocystis carinii infection, is an obscure protozoanrelated disease. The organism seems to be associated with stagnant water and may be a natural parasite of fish or other aquatic life.

ADIOSPIROMYCOSIS

This is a pulmonary disease found in many species of rodents throughout the world. It has rarely been described in man. The spores (2 to 4 μ) of the soil fungus are inhaled into the lungs, where they enlarge to a huge spherule. Development stops at this point and no endospores or buds are produced. The name adiospores refers to the enlargement and arrest of a fungal spore.

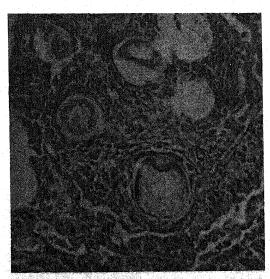


Figure 212. Adiospiromycosis spherules in the rat lung. Note the absence of endospores.

There is little host reaction. Adiospores can be produced in vitro by incubation at 40° C.⁴⁷ The fungi involved were described as two species by Emmons and named Emmonsia parva (Haplosporangium) and E. crescens. E. parva produces spherules up to 40μ ; E. crescens up to 500μ . Otherwise there is no difference in them. The organism is a soil saprophyte and grows on medium as a fluffy white colony producing single small (3 to 7μ) conidia on a conidiophore. Some authors classify them with Blastomyces dermatitidis in the genus Chrysosporium.

PARACOCCIDIOIDOMYCOSIS

The disease known as paracoccidioidal granuloma, Lutz's disease, or South American blastomycosis occurs in Brazil and elsewhere in South America and in Africa¹⁹³ but has not yet been observed in this country. The causative organism is Paracoccidioides brasiliensis which, despite its name, is not closely related to C. immitis but may be related to B. dermatitidis; it is also known as Blastomyces brasiliensis. It is a veast-like fungus, proliferating by budding in the tissues, and grows in a compact cerebriform colony on Sabouraud's agar, eventually producing a white aerial mycelium and single or double conidia shaped similar to those of B. dermatitidis.

The disease is clinically similar to coccidioidomycosis and blastomycosis.147 The fungus enters the body via the mouth to produce ulcerative granulomatous lesions which spread to the tongue, lips, and nose. The infection spreads via the lymphatics to produce lesions of the viscera and, often, pulmonary involvement. It is often fatal. It may also be acquired by inhalation, but rarely. Guinea pigs are usually infected by intratesticular inoculation and may also be infected by intratracheal inoculation. The fungus will grow readily on the chorioallantoic membrane of the embryonated hen's egg. There is a fluorescent antibody diagnostic technique described.205

Diagnosis is established by microscopic demonstration of the multiple budding cells (in contrast to the singly budding cells of B. dermatitidis) in material from the lesions or by culture. Treatment formerly consisted of 4 gm. of sulfadiazine a day for life, but amphotericin B offers hope of clinical cures.

Keloid blastomycosis. This disease, also

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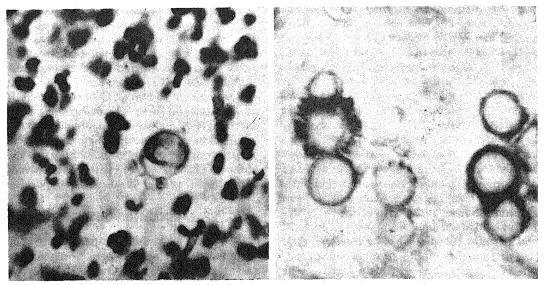


Figure 213. Left, giant yeast cell of Paracoccidioides brasiliensis showing multiple buds in skin section. Periodic acid-Schiff hematoxylin; × 400. Right, keloid blastomycosis. Chains of round cells in skin. Gridley stain; × 400.

called Lobo's disease, has also been described in South America. It is characterized by a budding yeast in chain-like formation. The tissue reaction is exaggerated fibrous hyperplasia leading to keloid formation. The organism called *Loboa loboi* has not as yet been cultured.^{21, 229}

HISTOPLASMOSIS165, 223

This organism was discovered in 1906 in Panama by Darling, who observed it in sections of tissues taken postmortem from three cases of what appeared to be visceral leishmaniasis and named it Histoplasma capsulatum. The first case in the United States was reported in 1926. The actual number of infections is undoubtedly many more than is indicated by reports of cases. Healed lesions of pulmonary infections are frequently found at autopsy in areas where the disease is prevalent,4 and the prevalence of the infection is shown by skin testing for hypersensitivity to the substance of the microorganism.70 It is now believed that in the United States 30 million people have or have had histoplasmosis; most in the Ohio and Mississippi River valley areas.4,72 The disease has been studied most intensively in this country, but apparently its distribution is world-wide. 42, 161

The causative organism. The organism appears in the tissues as a small, oval, yeast-like cell 2 to 4 μ in diameter with the ap-

pearance of a capsule, although there is none.41 In tissue stained with Giemsa stain it is difficult to distinguish the organism from that of leismaniasis except for the karyosome bar in the latter. Larger strains, up to 15 µ in diameter, are found in African histoplasmosis¹⁷³ (H. duboisii) and have been observed on occasion in this country. In stained preparations the central stained mass is surrounded by a clear zone. The organism is usually intracellular, in the mononuclears in the peripheral blood and in the macrophages elsewhere, especially those of the bone marrow and spleen. Because of this characteristic intracellular position of the parasite, the disease is sometimes called reticulo-endothelial cytomycosis or simply cytomycosis. 112 The common world-wide histoplasmosis is caused by H. capsulatum. The African form differs by its larger yeast form (H. duboisii). A third species, H. farciminosum, causes farcy in horses and is found around the Mediterranean area. It differs from the other two in having smooth chlamydospores in its saprophytic stage. The perfect stage of H. capsulatum is called Gymnoascus monbreuneii and is closely related to the dermatophytes.

The microorganism is cultivable from the blood or biopsy specimens, or it may be isolated from the sputum in pulmonary infections by animal inoculation during the course of the disease, and from various organs and tissues at autopsy in fatal cases.

It grows in the yeast-like form seen in the tissues in sealed blood agar cultures at 37° C. and in tissue culture, $^{111, 113, 139}$ but on Sabouraud's cycloheximide agar a mycelial form is assumed and the colonies are mold-like, white, and cottony with aerial mycelium when incubated at 25° C. 156 Chlamydospores (macroconidia) are formed in abundance, at first smooth and pyriform, but as they mature they become larger, 7 to $15~\mu$ in length, thickwalled, and tuberculate with finger-like protuberances sometimes as much as $6~\mu$ long.

Pathogenicity for man. Three types of diseases are generally recognized.29 The acute pulmonary type is characterized by sudden onset of malaise, fever, cough, chest pains, chills, sweats, and dyspnea, ranging in severity from severe flu syndrome to subclinical involvement. In this most common form the attack is followed by quick recovery and a fairly strong immunity. Progressive disease occurs in only 0.1 to 0.2 per cent of cases. The focal granuloma is associated with lymphadenitis of the hilar lymph nodes which then heals, often with calcification, and leaves a lesion roentgenographically identical to those of tuberculosis.221 In all its forms, histoplasmosis mimics tuberculosis.

The second type of disease, chronic progressive pulmonary histoplasmosis, may present as an exaggeration of the symptoms given above. It is usually found in middleaged males and is heralded by a persistent

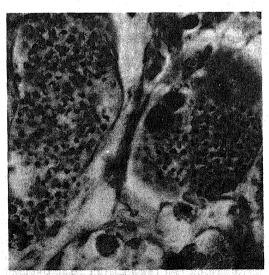


Figure 214. Histoplasma capsulatum. Yeast-like cells in macrophages of the bone marrow in systemic histoplasmosis. Hematoxylin and eosin; × 1000. (Humphreys.)

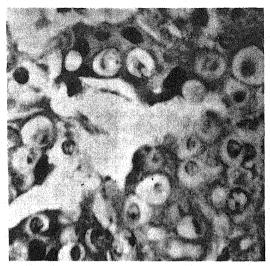


Figure 215. Giant yeast cells. of *Histoplasma duboisii*. African histoplasmosis. Hematoxylin and eosin; × 800.

complement fixation test. The disease progresses, like tuberculosis, to necrosis, caseation, and cavitation. The process is usually very slow.

The third form of the disease is disseminated histoplasmosis, which may develop at any age, but is commonly seen in infants or children. It has a rapid, fulminating, fatal course. Affected children are usually physiologically defective. This form of the disease is also seen in older people, particularly poor-risk patients, who may have lymphoma, Hodgkin's disease, diabetes, etc. The third patient-type seen is the patient with an old pulmonary infection which disseminates either by extension or reinfection. Dissemination may involve any organ, but especially the spleen and liver. The first clinical signs may be lesions on mucocutaneous areas.

Though it is sometimes implied that histoplasmosis is always a generalized infection of the macrophage system, the parasites being especially numerous in tissues rich in these cells such as the spleen and bone marrow, this is by no means generally true. In most cases there are nodules or extensive areas of necrosis in one or more organs, but in some instances only a single organ, such as the adrenals, has been infected. These necrotic lesions usually consist of a central area of necrosis surrounded by granulation tissue containing large numbers of macrophages and ingested parasites. In some instances the parasite has been found to be limited to such areas, while in others it is

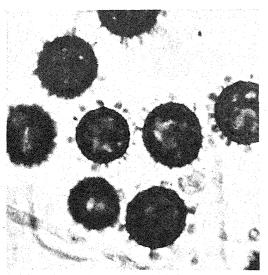


Figure 216. H. capsulatum. Mycelial stage showing tuberculate macroconidia (chlamydospore) and microconidia. Lactophenol-cotton blue; × 400.

widely distributed in the macrophage system as well. Infection of the central nervous system is relatively rare but has been observed. The statistical distribution of the lesions of the disease has been summarized by Schulz.²⁰⁰

Experimentally infected animals give a positive reaction to the intradermal inoculation of a preparation of liquid culture of H. capsulatum analogous to old tuberculin and coccidioidin and designated histoplasmin. In areas in which the disease is endemic, positive reactions are correlated with the incidence of pulmonary calcification in tuberculin-negative individuals. The association of positive histoplasmin reactions with calcified nodules in the lungs suggests that, as in the case of coccidioidomycosis, histoplasmosis is not a rare, highly fatal disease, but one which is widespread in certain geographical areas in a mild pulmonary form. That such reactors are or have been actually infected is difficult to demonstrate on any large scale, but infection has been shown in a sufficient number of cases to strongly support such an inference. Precipitins are produced early in the disease and disappear fairly rapidly.172 The complementfixing antibody titer rises somewhat later and disappears later, usually by 5 to 7 months. If the complement-fixing titer persists or rises, the prognosis is poor. Precipitating antibodies demonstable by the gel diffusion technique are also present. Two lines may be formed: the so-called M line, near the antigen well, indicates experience with the disease, and the thin H line nearer to the serum well correlates with active disease. 199 The fluorescent antibody technique is useful in the diagnosis of histoplasmosis. 129

The treatment of choice is amphotericin B in the usual course given for systemic fungous disease, 1 mg./kg. body weight to a total of 1 to 5 gm. The agent X5079C, now called Saramycin, holds hope of being a better overall therapeutic agent. There are some severe side affects from amphotericin B therapy. 15, 25, 44, 108

Pathogenicity for lower animals. A variety of animals have been found to be naturally infected, including dogs and cats, wild rodents, and other wild animals.45 An association with bats and bat caves⁴⁶ has been well established. The microorganism has been isolated from bat tissue.204 but the role of these animals as a reservoir and in the dissemination of the infection remains to be determined. It is improbable that these represent an animal reservoir of human infection; rather the fungus appears to be a saprophytic form, living in the soil from which it has been frequently isolated, which infects both man and animals. Mice have, in fact, been infected experimentally by exposure to soil contaminated with the microorganism.¹⁰⁷ Experimental animals are infected with some facility, a localized lesion being produced at the site of subcutaneous inoculation in guinea pigs and rabbits and generalized infection in dogs and rats. Both yeast-like and mycelial forms are infectious experimentally.

Epidemiology. The infection is airborne and is contracted by inhalation. Infection is associated with point sources such as chicken coops, pigeon roosts, starling roosts, bat-infested caves, and old decaying wooden structures where excreta and other decomposing organic matter under conditions of high humidity provide a nidus of infection. The infection tends to occur in rural areas and in low-lying river country. There is a large endemic area of infection in this country in the area including Missouri, Tennessee, Kentucky, Arkansas, adjacent areas in adjoining states, in which the proportion of positive reactors to the histoplasmin test ranges from 50 to over 90 per cent. This is in sharp contrast with the area from the Great Lakes to the Pacific coast and from Colorado to the Canadian border, in which the proportion of reactors is less than 2 per cent. Within the endemic area the proportion of reactors increases with age, from 5 per cent at two years, through 60 per cent at 18 years, to 75 to 90 per cent at age 55 and over.

Histoplasmosis is found in Africa, where it is known as cave disease because it has often been contracted in caves infested with bats. It differs slightly from American histoplasmosis. 163 Only sporadic cases have been reported from Europe. Consistent with this, the reactor rates are less than 2 per cent except in occasional isolated areas, such as Burma and northern Italy, where rates as high as 20 per cent have been observed.

Diagnosis. Microscopic demonstration of the fungus is highly suggestive of the disease, but culture and identification are reguired to establish the diagnosis. H. capsulatum has been isolated by blood culture in generalized infections and from biopsy specimens; sternal puncture may prove useful. It may be isolated fairly easily from sputum in cases of pulmonary infection by intraperitoneal inoculation of mice. The recommended procedure is to put the specimen on cycloheximide blood-agar and incubate at 25° C. Examination for characteristic tuberculate chlamydospores and demonstration of dimorphism at 37° C. establishes the identity of the organism.

OTHER SYSTEMIC MYCOSES

CRYPTOCOCCOSIS143

In medical literature the term blastomy-cosis has been used very loosely to designate etiologically diverse infections in which budding cells are found in the tissues. Confusion arises largely between European blastomycosis, which will be considered here, and American blastomycosis, taken up in a previous section.

Aside from candidiasis, two general types of yeast infection may be distinguished, the one with deep-seated cutaneous or subcutaneous infections which tend to become generalized, and the other with infections of the central nervous system arising, as a rule, by metastasis from foci in the lungs. The first of these is known as European blastomycosis and the second as torula meningitis. Both are caused by the pathogenic yeast Cryptococcus neoformans.

European blastomycosis. What was probably the first case of yeast infection of proved etiology was a fatal generalized infection observed by Busse and by Buschke in 1893, which they called systemic blastomycosis and which subsequently has been generally known as European blastomycosis. From primary ulcers on the face and neck, the infection spreads to the cervical lymph nodes, and the causative organism was isolated first from a secondary tibial abscess, then from the primary ulcers, and shortly before death from the blood stream. Other cases reported since are of this general type, characterized by deep-seated ulceration of the skin, 166 sometimes granulomatous, and there may be infection of the viscera, usually secondary, involving the spleen, liver, kidneys, and mesenteric lymphatics. Infection of the lungs may be primary, may occasionally be arrested with no further manifestations, or may spread from the focus in the lungs; lung infection may also be secondary.

The microorganisms are found in exudates and in mucoid masses of gelatinous material as round to oval cells, 5 to 6 μ in diameter, surrounded by a mucilaginous sheath. The gelatinous material in which they may be embedded is evidently a product of the fungus. It is reported that the cells may be stained specifically in tissue sections and smears by the fluorescent antibody technique. They are readily cultivated on most ordinary mediums as a smooth white or very light tan colony without distinguishing features. The organism will not grow on mediums containing cycloheximide.

Cryptococcus meningitis. A number of cases of yeast etiology have been reported, the great majority from this country. The clinical features of the disease set it off from European blastomycosis, for the infection is predominantly one of the central nervous system, and the skin is seldom involved. The symptoms are those of brain involvement, especially intracranial pressure. Brain tumor may be closely simulated in some cases, and the disease develops slowly, usually without febrile reaction or other signs of infection; a case of 16 years' duration has been reported. The pathological picture is that of chronic leptomeningitis with thickened meninges adherent to the cerebral cortex and showing diffuse or focal granulomatous lesions. The cerebral cortex is invaded in about half the cases; the lesions are sometimes granulomatous but

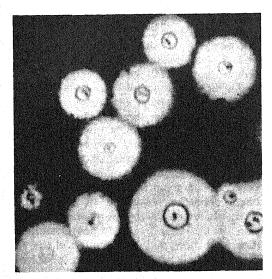


Figure 217. India ink preparation of *Cryptococcus* neoformans. Yeast cell in center surrounded by capsule. × 800.

more often cystic, and there is little if any inflammatory reaction. The granulomatous lesions of both meninges and brain contain large accumulations of macrophages which phagocytose the fungus, while the cystic lesions consist of enormous numbers of yeast cells embedded in a gelatinous matrix. The yeast is usually present in the spinal fluid in pure culture and may be observed in wet unstained preparations of the centrifuged sediment in a drop of India ink. The lung and kidney are the next most frequently involved organs, but any tissue may be attacked, with generalization of the infection.¹⁸ There is a strong association of cryptococcal infection with such debilitating diseases as lymphomas and leukemias, and such patients run a particular danger of a rapidly fatal infection. There is reason to believe that infection is more common than has been supposed, perhaps 2000 for every proven case, and that the portal of entry is the lung. Unlike histoplasmosis, blastomycosis or coccidioidomycosis, there is so little tissue reaction that a skin test has not as yet been developed. Current estimates indicate 15,000 subclinical or clinical cases per year in New York City alone. It is almost always fatal in untreated cases, but amphotericin B therapy has been reported to be effective. 11, 71, 175

Pathogenicity for lower animals. Infection occurs in lower animals, notably in the lungs and nasal granulomata of horses, and

cryptococcus has been described as the etiological agent in a severe outbreak of bovine mastitis, but there is no reason to believe that lower animals constitute a reservoir of human infection. The organism has been isolated from soil,3 and it seems probable that this is the source of both human and animal infections. There is a close association of the organism with pigeon droppings. 124, 142 In areas crusted with bird dung, such as sills, ledges, attics (but infrequently in enriched soil), the organisms can easily be found if looked for, and in large numbers. Point source infections have been documented in New York¹⁴² and Chicago¹⁷⁶ as well as many other places.232 The organism does not appear to be in the pigeon body itself, just in the filth left by the bird. The microorganism is virulent for mice with an LD₅₀ of about 1000 cells on intracerebral inoculation; following intraperitoneal inoculation it produces a generalized infection that spreads to the central nervous system. Experimental infection has also been produced in marmosets (Leontocebus geoffroyi) by feeding them large numbers of the microorganisms.225 Some immunity has been achieved in experimental animals,1 but effective antibodies in human infections are highly debatable.^{73, 74} Serological procedures for diagnosis are not reliable, except for the fluorescent antibody technique on tissue sections. 128 A nonantibody anticryptococcal

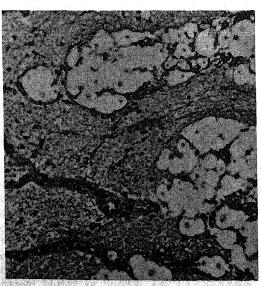


Figure 218. C. neoformans in optic nerve. Spaces represent areas occupied by capsular material. Periodic acid-Schiff hematoxylin; × 400.

factor in human serum has been reported. 114 A skin test is being developed. 194

The causative organism. For some time the relationship of these clinically diverse infections was not realized, and the yeast of European blastomycosis was known as C. hominis and that of torula meningitis as T. histolytica. In 1934, however, Benham showed that strains of the two yeasts were substantially identical, the difference in pathogenicity being one of degree only. Similar observations have been reported by others. It seems established, therefore, that these diseases are caused by the same etiological agent and, furthermore, that only a single species of yeast is involved, observed strain differences having no more than variant status.

It characteristically assimilates (but does not ferment) glucose and sucrose, not lactose or melibiose. It is the only member of the genus pathogenic for mice. The genus members characteristically produce urease and this, together with their lack of mycelium production, separates them from the genus Candida. C. neoformans (C. hominis, Debaromyces neoformans, Torula histolytica) is the only pathogen in the genus. A nonpathogenic variety, C. neoformans var. innocuus, and several other species, including C. laurentii, C. mucorugosus, and C. luteolus, are accepted as valid species,16 but differ from C. neoformans in the above physiological tests. The heavily encapsulated forms are poor immunizing antigens, but satisfactory antiserums may be prepared in the rabbit with poorly encapsulated forms. The organism gives a Quellung reaction in homologous antiserum. There are at least three immunological types, designated A, B, and C by Evans, 57, 58, 59 depending upon the polysaccharide capsular antigen. The polysaccharides are closely related chemically, all containing xylose, mannose, galactose, and a uronic acid.

Torulopsosis. Torulopsis glabrata was isolated from animal infection in 1939. It is a small (3 to 4μ) intracellular parasite, and infection in tissue somewhat resembles histoplasmosis. The organism is fairly commonly isolated from urine and may be involved in infections. ¹²¹ It has been isolated from uncomplicated meningitis as the cause of death. The organism is like Cryptococcus, always in a yeast form. It produces a pasty white to gray colony, ferments glucose (acid and gas), and assimilates only glucose. A similar species, T. pintolopesii is found in

the mouse alimentary tract and may lead to confusion in experimental infection by human pathogenic fungi.

Geotrichosis. Geotrichosis is an infection of the oral, intestinal, bronchial, or pulmonary system by a semiyeast organism, Geotrichum candidum.28 The nomenclatural synonomy and relationships, even if there is only one species, are much debated. Geotrichosis seems to have been differentiated from candidiasis in 1842. Bronchial geotrichosis is a rare but fairly well delineated entity. Chronic bronchitis with persistent expectoration of mucoid sputum and with little or no rise in temperature is the general symptomatology. Oral geotrichosis resembles oral candidiasis, as does the rarely reported beer yeast (Saccharomyces cerevisiae) oral and bronchial infections. Other sites of infection have been reported, but the status is provisional as the organism is a common contaminant. It is easily isolated from feces, cottage cheese, and tomatoes. The colony is a white, mealy, and barrelshaped arthrospore similar to Coccidioides (but lacking the empty space between cells) and can be seen under the microscope.

ASPERGILLOSIS

The common blue-green mold seen on damp bread, bacon, or most any organic material is usually a member of the genus Aspergillus. The other common blue-green fungus is Penicillium species. Together these organisms constitute the most omnipresent and ubiquitously distributed molds in the world. With every breath we inhale some spores of one or more species. Most of the time these spores are disposed of without injury to the host. In some individuals and under certain conditions, these organisms may provoke injury to man in one of two ways: allergic response to the presence of the spore or, much more rarely, invasion of pulmonary or other tissue.

Of the two groups, Aspergillus is more commonly associated with infection than Penicillium, and one species, A. fumigatus, is highly pathogenic for birds and also occasionally for man. There are over 70 species of the genus Aspergillus, of which a dozen or more have been described as responsible for infection. Of the more than 140 species of Penicillium only a few have been isolated from the very rare disease penicilliosis. Though the genus names are

in the family Moniliaceae, order Moniliales of the Fungi Imperfecti, a few of each genus produce a sexual stage. The sexual spore is an ascus and the group, therefore, is in the class Ascomycetes, order Eurotiales.

Pathogenicity for lower animals. Aspergillosis of domesticated birds, pigeons, ducks, penguins, and chickens is not uncommon and at times assumes economic importance. Three types of infection commonly occur; infection of the air sacs and both nodular and pneumonic forms of lung infection. A fourth type, aspergillar meningitis, is found with some frequency in certain host groups, i.e., penguins. Infection of the air sacs takes the form of a superficial infection of the epithelial lining, which becomes thickened and covered with a mat of green sporulating mycelium. In the nodular form of the disease, tubercle-like masses of infiltrated tissue, necrotic in the center, are formed. A diffuse infiltrate is formed in the pneumonic form, and the lung tissue is consolidated and grayish white in color. The pneumonic disease sometimes assumes epidemic form in chicks and is known as "brooder pneumonia." The source of infection is usually moldy grain or straw. The fungus may also invade the egg during incubation, with infection of the embryo. Cattle, sheep, and especially horses develop aspergillosis, though less commonly than birds. The lesions are pulmonary and may be either nodular or pneumonic.

Strains of infectious aspergilli vary widely in their pathogenicity when tested by inoculation of experimental animals. In general, those isolated from infections are quite virulent, while those found in air and elsewhere are of low virulence. With virulent strains a fulminating, rapidly fatal pneumonia may be produced in pigeons by inhalation of spores, while the feeding of grain overgrown with the mold often produces an infection of the air sacs. Intravenous inoculation of pigeons results in an acute infection with multiple miliary abscesses, especially in the lungs, if the dose is not too large. Otherwise, rapid toxic death occurs. Rabbits inoculated intravenously with spore suspensions of virulent strains usually die in three to five days, with multiple abscess formation, notably in the kidney, whereas subcutaneous or intraperitoneal inoculation produces localized lesions which may or may not be fatal. The subject of Aspergillus pathogenicity is one of the most enigmatic in the study of the fungi. Some isolates of a species can be injected in large numbers and produce no disease; other isolates of the same species are as virulent or more so than *Coccidioides immitis*. Many Aspergillus and Penicillium species can be induced into a yeast-like growth similar to that of *Blastomyces dermatitidis* in which they are very invasive for the deep organs of experimental animals.¹⁸⁰

Pathogenicity for man. Human cases of aspergillosis are most frequently infections of the external ear (otomycosis). The disease varies from a plugging of the external meatus with a mass of mycelium to ulceration of the walls and even penetration of the middle ear; the mild cases are the most numerous. It is fascinating to look through an otoscope and see the external auditory canal lined with the delicate, beautifully architectured fruiting heads of an Aspergillus. While A. fumigatus is the most common invader, A. nidulans, A. flavus, and A. niger are sometimes found.

Pulmonary infection is relatively uncommon in man, more cases appearing in Europe than in the United States. It occurs in two forms, the first, an intracavitary fungous mass, "fungous ball" or aspergilloma, 186 and the second, bronchopneumonic aspergillosis. 30, 85, 164 In the former, there is little frank invasion of living tissue, and the disease may manifest itself as an extreme allergic response to the presence of the mold. In the latter type, invasion of tissue and more serious disease occur, frequently resulting in death. Infections of both types may be secondary to tuberculosis, other pulmonary fungous diseases, neoplasms, or cytopathic drugs. It is of some interest that the primary infection has been classed as an occupational disease with regard to compensation. The disease is clinically similar to and may be mistaken for tuberculosis, neoplasm, or histoplasmosis; there may be extensive cavitation and it may advance rapidly. The mycelium may be found in the sputum and the fungus is readily isolated by culture (not on cycloheximide agar) but all other etiological agents must be excluded and great caution must be exercised before attributing an infection to such common contaminants as aspergilli. 109 Many lung infections have been reported in grain handlers, millworkers, and bird-fanciers, especially those having to do with the care of pigeons; there is convincing evidence that transmission of the disease from pigeons to man can take place.

Very rarely aspergillosis may take other forms in man. The occurrence of Aspergillus in maduromycosis has been noted, and a case of chronic suppuration with discharge of grains has been reported. Rarely it may assume an acute disseminated form, including involvement of the central nervous system. 94 It is not infrequently isolated as the cause of keratitis of the lens following a puncture wound of the eye. A precipitin test has been developed. 218

A final word must be said about the constant danger of aspergilli and other common soil fungi to debilitated patients. A. fumagatus is a thermophilic fungus, growing well at 37° C., and it also produces a potent endotoxin. 104 It and many other saprophytes readily invade patients on cytopathic drugs, nitrogen mustard, antimetabolites, or antimune drugs (Imuran) or patients with debilitating diseases, particularly neoplasms, less so diabetes. 97 Many organ-transplant failures have resulted from infection by "saprophytes." 224 Treatment of choice is amphotericin B. 174

MUCORMYCOSIS 157

Mucormycosis has been a very rare infection, occurring largely as a mycotic complication of chronic debilitating disease, most often uncontrolled diabetes but also amebic colitis and kwashiorkor.^{48, 228} With the introduction into general use of therapeutic agents such as antibiotics, corticosteroids, and antileukemic drugs the infection has been found more often, so much so that it is considered to be a "new disease."¹⁰

The fungi, usually species of Rhizopus, are apparently of very low virulence and are able to invade the tissues only when natural resistance is markedly reduced. Infection usually begins in the upper respiratory tract, most often the nose, where spores germinate and the mycelial growth invades the mucous membrane and may extend into the adjacent sinuses, orbital cavity, or cerebral tissue.87 Primary infection may also occur in the lung, where growth on the bronchial mucosa penetrates the wall to the hilar tissue or a lobar pneumonia may result. The fungus appears to have a special affinity for arteries, with penetration into the lumen to produce thrombosis and infarction,



Figure 219. Mucormycosis showing broad nonseptate mycelium in brain tissue. Periodic acid-Schiff hematoxylin; × 800.

and may reach the central nervous system via ophthalmic and internal carotid arteries to produce a meningo-encephalitis. In tissue the organism appears as broad, distorted, nonseptate hyphae. Most cases are diagnosed at autopsy; the tissue having been thoroughly soaked in formaldehyde and no cultures obtainable. Most species grow readily on mediums without cycloheximide. Members of the genera Mucor, Absidia, and Rhizopus have most often been isolated. They are members of the order Mucorales of the class Phycomycetes. Other phycomycete diseases have been discussed and, in order not to confuse the entities, the proposed change of name to phycomycosis is not followed. The clinical pictures are quite different.122

MISCELLANEOUS MYCOSES

Various other fungi have occasionally been described as producing human disease. Though hundreds of fungus species have been recorded as isolated from lesions, it is quite difficult to assess their significance in the disease process. If the organism is isolated more than once and in quantity from exposed lesions or sputa and all other etiologies have been ruled out, it may be considered as responsible. However, if the organism is isolated from a closed body cavity, lesion, or blood culture, it must be

considered as a strong suspect in the disease process. Below are listed some rarely encountered fungous diseases in which fungi have been isolated with sufficient frequency to establish their potential invasive ability.

Penicilliosis. The ubiquity of species of Pencillium and their constant contamination of instruments, wounds, urine, sputum, etc., makes the establishment of this diagnosis very difficult. P. marneffei is found as a veast-like invasive pathogen of Asian rodents. Substantiated infection of the cornea and the external ear, a few mycetomas, and a very rare pulmonary infection are recorded for a variety of Penicillium species. The pathological picture is similar to aspergillosis. The related genus Scopulariopsis, especially S. brevicaulis, is a not uncommon agent of onychomycosis and peronychia, characterized by much inflammation and pus.

Cercosporamycosis. Cercospora apii,⁵¹ a common plant pathogen (black streak of onion, celery blight), has been the cause of a subcutaneous, indurated, verrucous infectious process. Brown mycelium is seen in tissue. The cases reported thus far have been from Indonesia.

Cladosporiosis. Species of the genus cladosporium, especially C. trichoides, have been isolated several times from cerebral and pulmonary lesions. Many cases have been fatal, and the disease is called black degeneration of the brain for its most apparent pathological picture. The lesions usually are localized and encapsulated. In tissue sections, multiseptate, brown, distorted hyphae are seen. The organism (a Dematiaceae, Fungi Imperfecti) grows well as a black velvety colony on mediums without cycloheximide. It is pathogenic for experimental animals and is neurotropic. 130

Mycotic infections of the eye. Introduction of fungal spores into the cornea by abrasion or secondary to herpetic lesions is not an uncommon occurrence. Establishment of the infection is enhanced by the overuse of steroids.56 The organisms involved are most often soil saprophytes, such as Aspergillus, Fusarium, and Hormodendrum.80 The infection is usually localized but may destroy the cornea or spread to the rest of the eye. Hematogenous spread of pathogenic fungi from other loci to the eye is known but is very rare. A condition, histoplasma uveitis, is recognized by ophthalmologists, but a causal relationship to Histoplasma capsulatum has yet to be demonstrated. 192, 215 Treatment of corneal infection is usually with irrigation of Nystatin or amphotericin B solutions.

MYCOTOXICOSIS

The ingestion of particular fungi or products elaborated by particular fungi sometimes leads to a variety of distresses to the individual. The distress may range from nausea (Russula emetica),13 hallucination (Psilocybe species), and severe dermatitis (Pithomyces species) to carcinoma (Aflatoxin) and death (Amanita phalloides). The fatal toxicity of certain mushrooms was well known in ancient times. Agrippina was familiar with several poisonous agarics and the dosages necessary for the desired end. More recently, the endotoxins of aspergilli were investigated by Henrici, in the 1930's. At present there is renewed interest in this subject because of the potential carcinogen found in foodstuffs infected with Aspergillus flavus and the recreational use of hallucinogenic drugs such as psilocybin and d-lysergic acid (from Claviceps purpurea).

Aflatoxin. A chloroform-soluble, small molecular weight substance elaborated by Aspergillus flavus and A. parasiticus known as aflatoxin is regularly able to induce hepatomas in ducklings and rats. 170 There have been widespread epidemics of high mortality among stock animals following ingestion of moldy feed, particularly peanuts, corn, or peas. The disease has been reported from all parts of the world and affects a variety of animals; cattle in Africa, "X" disease of turkeys, etc. The toxin has an empirical formula of C₁₇H₁₂O₆ and is structurally related to synthetic coumarin with a dihydrofuran substitution. It can be detected easily by characteristic fluorescence and by hepatoxic changes in ducklings. Embryonic lung cell tissue culture can detect as little as 0.01

 μ g. of aflatoxin B.

Mushroom poisoning. Ford in 1923 recognized five types of mushroom poisoning which appear to be still valid. 49

Mycetismus gastrointestinalis. The chief complaint in this mildest form of mycotoxicosis is nausea, which may be followed by vomiting and diarrhea. Spontaneous recovery usually occurs in one to two days. This type of "poisoning" is particularly

common in children following ingestion of the orange jack-o-lantern mushroom, Clitocybe illudens. Many other species will produce the same syndrome, for example, Russala emetica, Boletus satanas and other boletes, Entoloma lividum, and Lepiota morgani. L. morgani has been responsible for a few deaths.

Mycetismus choleriformis. This is the most serious and often fatal type of intoxication. The death angel (Amanita phalloides), the destroying angel (Amanita verna), and probably other Amanita species produce a heat-stable, bicyclic hexapeptide, phalloidin. It is one of the most potent toxins known. The course of symptoms is similar to that in phosphorus intoxication and may appear six to 18 hours following ingestion. Abdominal pain, vomiting, diarrhea, bloody stool with mucous strands, protein and casts in urine, malaise, and cyanosis may have a fatal outcome in two to three days. Temporary remission and relapse may also occur. Treatment includes complete evacuation of the alimentary tract, opiates, intravenous glucose in saline, and rest. Hygrophorus conicus and Pholiota autumnalis may also induce a similar disease—usually milder.

Mycetismus nervosus. Muscarin is a heatstable, substituted quaternary ammonia (related to choline) which excites the parasympathetic system. It is found in Amanita patherina and A. muscaria. Symptoms occur within one to three hours after ingestion and may include tearing, sweating, salivation, retching, peristent peristalsis, vomiting, diarrhea, contraction of pupils and ciliary muscles, acute excitement, delirium, and coma. Treatment includes gastric lavage and atropine. These fungi have been used since ancient times in Sweden and Sibera as intoxicants. Inocybe infida, I. infelix, and Clitocybe illudens give milder types of a similar disease.

Mycetismus sanguinareus. A heat-stable hemolysin is found in Helvella esculenta and other Helvella species. Hemoglobinuria, abdominal distress, and jaundice may occur. Hemolysins are present in several fungi but are usually destroyed by digestion or heating.

Mycetismus cerebris. Hallucinogenic intoxication occurs following the ingestion of several fungi. The active principle is usually a small molecular weight substance related to indol (viz., d-lysergic acid, psilocybin). These psychodelic or psychotropic

mushrooms have been used for centuries in certain Mexican communities in religious rites. They have recently become popular as sensory- or perception-heightening agents among certain segments of society who refer to their use as a "trip"! Many species of the genera of the brown-spored mushrooms such as Paneolus, Psilocybe, Stropharia, and Conocybe may contain varying amounts and types of the hallucinogenic agents.

Many other types of toxic conditions may result from the ingestion of various fungi or their products. Ergot of rye (Claviceps purpurea) results in blackleg (a hemorrhagic disease), ergotism, confusion, and death following ingestion in flour or feed. Numerous pharmacologically active substances are produced by this organism, including lysergic acid and ergotamine. Stachybotrys toxicosis results from eating moldy forage infected with Stachybotrys alterans. Desquamation of epithelial linings, leukopenia and fever may lead to death in one to five days in severe cases. The disease is most prevalent in the Ukraine. Alimentary toxic aleukia may result from grain or forage infected with Fusarium sporotrichoides or F. roseum. The symptoms are similar to Stachybotrys toxicosis. A disease of sheep called facial eczema results from ingestion of the grass Sporidesmium bakeri infected with the mold Pithomyces chartarium. The active ingredient is sporidesmin, which has an empirical formula of $(C_{19}H_{21}O_6N_3S_2Cl$ C Cl₄). Red clover hay infected with Rhizotonia leguminicola causes excessive salivation in foraging animals.37 The active principle is a parasympathomimetic alkaloid. Many other toxins of varying importance have been described from many fungal species.

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Chapter Thirty-three

THE SPIROCHETES

Spiral shaped microorganisms were for a long time grouped together indifferently under the common name of spirillum, spirochete, or vibrio. Further knowledge has shown that some of these are true bacteria. although curved, and such forms are now placed with the bacteria in the genus Vibrio and various species of Spirillum. Others, however, possess characteristics which distinguish them from curved bacteria, such as the cholera vibrio, and also from the actively motile protozoa, such as the trypanosomes. Like the bacteria, the spirochetes multiply by transverse fission, have no well-defined nucleus, and do not exhibit anterior-posterior polarity. They appear to be more closely related to the true bacteria than to the protozoa and may, perhaps, be regarded as a connecting link between the two. 107

Classification and nomenclature within the group are still in an uncertain condition, and there is by no means general agreement regarding the separation of some of the genera. These microorganisms are difficult to cultivate on artificial mediums in many cases, and little or nothing is known of their physiological characteristics; the genera are separated from one another on a purely morphological basis.³⁰ Studies by electron microscopy have tended to confirm the soundness of the morphological criteria.¹⁰²

Although the name spirochete is commonly used to designate those forms which are classified as members of the family Spirochaetaceae, the generic term Spirochaeta was originally proposed by Ehrenberg for the large free-living forms he described. A second genus, Cristispira, is characterized by the presence of a membranous appendage or crista wound about the body of the cell and is made up of microorganisms found living as saprophytic commensals in certain molluscs. A third genus. Saprospira, consists of free-living forms which differ from Cristispira in that the crista is absent. The other genera contain the spirochetes which are pathogenic for man and animals: Borrelia, the spirochetes of the relapsing fevers; Leptospira, the spirochetes causing Weil's disease or infectious jaundice and certain fevers; and Treponema, the spirochetes of syphilis and vaws.

The Spirochetes of the Relapsing Fevers

(Borrelia)³⁷

The relapsing fevers are a group of closely related infections characterized clinically by an initial pyrexia of three to four days' duration, followed at intervals of a few days by successive relapses. They are widely distributed and occur in every country of the world with the possible exception of Australia. The microorganisms responsible

for these diseases are spiral forms, first observed by Obermeier in 1873 in the blood of patients with European relapsing fever.

Morphology. The basic structure of these spirochetes is a spring-like axial filament upon which there is a layer of contractile protoplasm enveloped in a delicate periplast. The terminal filaments are, per-

haps, drawn-out ends of the periplast or nonrigid and noncoiled ends of the axial filament. Attached spherical bodies are sometimes observed. The cell rotates as a consequence of the stretching of the axial filament by the pressure of contracting protoplasm. When there are rapid and successive contractions and relaxations, the microorganism rotates rapidly and moves in one direction or another according to the direction of the rotation. In the relaxed state the spirals are regular and even; the larger the cell the heavier the axial filament and the greater the distance between the spirals. The regularity of the spirals is disturbed by the contracting and relaxing protoplasm in the actively motile cells. The various species cannot be distinguished from one another on a morphological basis. All are 0.2 to 0.5 μ in breadth by 10 to 20 μ in length. They are best stained by the Romanowsky method or some modification such as Giemsa, but, in contrast to some of the other spirochetes, Borrelia may be stained with the ordinary aniline dyes.

Cultivation. The spirochetes of relapsing fever were cultivated by Noguchi in 1912 in a medium of ascitic fluid and fresh tissue in which a network of fibrin is formed. A semisolid serum agar has been used also. In culture the spirochetes are aerobic, and the paraffin-oil seal frequently used prevents evaporation rather than interferes with the diffusion of oxygen. While such successful cultivation has been reported, the spirochetes are exceedingly difficult to grow in initial culture and cannot be maintained in serial passage; it is not unlikely that they only persist for a time in such mediums and never have been actually cul-

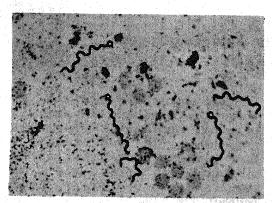


Figure 220. Borrelia recurrentis in a blood smear from an infected rat. Fontana stain; × 1160. (Freeman.)

tivated. They may be grown in the developing chick embryo, however, and have been isolated directly from human infections in this way.^{14, 85}

Classification. The relapsing fever spirochetes have been given the generic name Borrelia. The validity of this degree of differentiation from the spirochetes of syphilis and yaws, Treponema, is open to some question, and the relapsing fever spirochetes are regarded by many as Treponema species. It was pointed out by Noguchi that the relapsing fever spirochetes do not differ more from *Treponema pertenue* (yaws) than that microorganism differs from *T. pallidum*.

It is not clear to what extent the relapsing fever spirochetes can be divided into species. It is probable that in many cases those given species rank are, in fact, only strains or varieties. In many instances these have been given place names or named after some individual worker, but the differentiation has been geographical rather than biological. These forms are immunologically heterogeneous and unstable in that they are modified in character by residence in different insect or mammalian hosts. Cross-immunity tests, therefore, are not useful. It has been suggested that they are differentiated on the basis of pathogenicity or insect host, but there appear to be no reasonable definite lines of demarcation possible on these bases. In general, then, the questions of interrelationship and speciation of these organisms are almost completely open but most workers agree that, although they are all immunologically related, there is too great heterogeneity to justify regarding the various strains as no more than varieties of a single species Borrelia (Treponema) recurrentis.

Pathogenicity. Relapsing fever is an ancient disease, and one of the first epidemics was described by Hipprocrates as occurring on the island of Thasos 2000 years ago. It was not mentioned for many centures, and then there was a tendency to confuse it with louseborne typhus fever with which it may occur. The Yellow Plague that followed the pandemic of bubonic plague in the time of Justinian was probably relapsing fever, and an epidemic in Ireland in 664 was described by the Venerable Bede. At the present time it occurs sporadically in various parts of the world.

As indicated above, the relapsing fevers constitute a group of closely related dis-

BORRELIA 747

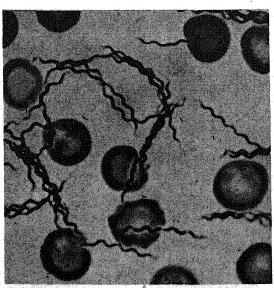
eases. The spirochete observed by Obermeier was that of European relapsing fever. a disease which has been known since the early part of the eighteenth century and has at times prevailed extensively in parts of Europe. The causal microorganism is designated Borrelia (or Treponema) recurrentis. Four forms of relapsing fever occur in Africa; the spirochete of the form in Central Africa is B. duttonii, which is also found in the Middle East1; that of the East African disease B. kochii; that occurring in northern Africa,³¹ in Tunis, Algers, and Tripoli, is B. berbera; and that in the Sudan B. aegyptica, and Borrelia hispanica is found in Spain and North Africa. Relapsing fever also occurs in India, and the causative microorganism is B. carteri. American relapsing fever is caused by still another spirochete, B. novyi; other strains isolated in this country have been named B. turicatae. South American strains have been called B. venezuelensis and B. neotropicalis.

All forms of relapsing fever are clinically identical. The onset is sudden, with chills, fever, and severe headache; muscular and joint pains are frequently observed, and there is a moderate enlargement and tenderness of the spleen and commonly jaundice. The fever ends suddenly by crisis in three or four days. Successive relapses occur at intervals of two to 14 days, and the period of relapse varies from a few hours to longer than the primary fever. The number of re-

lapses varies. Spirochetes may be found in the blood during the paroxysms. The case fatality is not high in European relapsing fever, perhaps 4 to 5 per cent. There are no characteristic findings at autopsy.

The infection may be transmitted to the rat and mouse, but the guinea pig appears resistant to most strains though it may be infected with certain American strains. Neonatal guinea pigs have been found to be more susceptible, and the susceptibility is differential with respect to at least some strains.11 It has been reported that the hamster may be infected with at least some strains. It is of some importance that the blood for inoculation be taken at the onset rather than at the decline of the relapse. Ordinarily the infection is inapparent in both rats and mice in that there are no observable symptoms, but successive "attacks," beginning two to four days after inoculation, are evidenced by the appearance of the spirochetes in the blood. They are present for two to three days, disappear, and reappear three or four days later in a second "attack." Usually only two or three such relapses occur, the spirochetes becoming fewer and persisting for shorter times in the successive relapses. Experimental disease may also be produced in monkeys, and its course is very similar to that in man. There is some evidence that tetracyclines may have utility as chemotherapeutic agents.

Natural infection of lower animals occurs



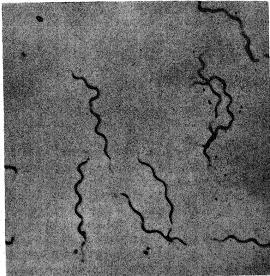


Figure 221. Relapsing fever spirochetes. Left, Borrelia duttonii of Central African relapsing fever; right, Borrelia kochii of East African relapsing fever. × 2000. (Kral.)

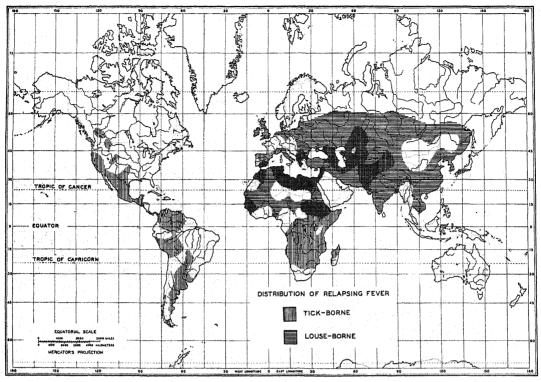


Figure 222. The world-wide distribution of relapsing fever, both louse- and tickborne. (Redrawn from map prepared by Army Medical Intelligence, 1943. Based on Goode Base Map No. 201 M. By permission of the University of Chicago Press.)

with some frequency in endemic areas, and there is much evidence to support the hypothesis that small mammals, especially rodents, constitute a natural reservoir of the tickborne infection. A wide variety of animals may be infected, including the rat, mouse, chipmunk, squirrel, opossum, porcupine, armadillo, hedgehog, fox, and dog. Susceptibility varies considerably among animal species, and from one strain to another of the spirochete.

A spirochetosis of geese is due to B. anserinum, a microorganism regarded by many as identical with B. gallinarum, which produces septicemia of chickens. Another species, B. theileri, is associated with a benign affection of cattle in South Africa but appears to have but low pathogenic powers. Morphologically similar spirochetes have also been found in the blood of sheep, horses, and bats and in the alimentary tract of fish and insects, but they are not associated with any obvious indisposition.

Immunity. Following infection, agglutinins and lytic and spirocheticidal antibodies may be found in the serum. Usually

the immunity following recovery from an attack of relapsing fever is of short duration, but occasionally a more solid immunity may develop. The latter is often attributed to a persistence of the infection, *i.e.*, an immunity to superinfection.

Relapse appears to be basically an immunological phenomenon and dependent upon the antigenic instability of the spirochete. It has been shown that the spirochetes will remain alive for as long as 40 days in blood drawn before the onset of an attack, but in blood drawn during the decline of an attack or after recovery they die out in less than an hour. In the latter case the blood is spirocheticidal and the killed spirochetes are phagocytosed. This has been taken to suggest that the relapse is a consequence of the survival of a few individuals which are resistant to the specific spirocheticidin and which multiply to give rise to a new serumfast strain. It has been found that spirochetes isolated after successive relapses differ serologically from the spirochetes of the first attack, and as many as nine different serological types of B. carteri were distinguished by Cunningham; after any particular type appeared in an animal it never reappeared in the further course of the disease in the same animal. It has been found that the two North American varieties, B. turicatae and B. parkeri, contain three antigenic components, A, B, and C, of which B is shared and invariant, while A and C are both strain and relapse-specific. Relapse serums are protective against both initial attack and relapse, whereas attack serum protects against attack but not relapse strains.

The parallel between this and the successive relapses of certain types of trypanosomiasis is striking (Chap. Thirty-four). In the guinea pig infected with Trypanosoma rhodesiense recovery is due to the development of a specific trypanolysin, the relapse to the multiplication of serum-fast trypanosomes, second recovery to a second specific trypanolysin, and so on. In this case the serum-fast strains are immunologically distinct from one another. In T. lewisi infections in the rat, however, both a trypanolysin and a reproduction-inhibiting antibody, or ablastin, are formed; the inhibition of cell division does not, presumably, allow the development of serum-fast strains, and hence there are no relapses.

Diagnosis. 15 The diagnosis of relapsing fever is made by demonstration of the spirochetes in the blood during the onset of a relapse by direct microscopic examination or by animal inoculation. Either the usual blood smear or a thick film similar to that used for the detection of malarial parasites may be used. The films are air-dried and stained with Giemsa; Wright's stain is satisfactory for thin films. The spirochetes may also be found in fresh wet preparations by darkfield examination. Mice and/or rats may be inoculated intraperitoneally and blood smears made daily for as long as two weeks if the spirochetes are not found.

Immunological methods of diagnosis have not been satisfactory in the past but Stein¹⁰¹ has prepared spirochete antigens by saponin hemolysis and washing of the spirochetes. Complement fixation, said to be preferable for diagnostic purposes, and agglutination with serums from infected persons or animals occur and are reported to be specific.

Epidemiology. The relapsing fever spirochetes are blood rather than tissue parasites, and the infection is transmitted by blood-

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sucking insects, though rare cases of direct transmission may possibly occur. Two epidemiological types of the disease are to be distinguished, the one tickborne and representing transmission from an animal reservoir of infection to man, and the other louseborne and spread from man to man.⁹⁹

A tick vector was not recognized in this country until 1930, but the tickborne disease is now known to be not uncommon and at the present time is the only type which occurs. Known endemic foci of infection exist in Arizona, California, Colorado, Idaho, Kansas, New Mexico, Nevada, Oklahoma, Oregon, and Texas and possibly also in Montana, Utah, and Washington. Ornithodorus turicata and O. hermsi are known to transmit the disease, and it seems probable that O. parkeri transmits it also. but final proof is lacking. O. talaje transmits the infection in tropical America but apparently not in the United States; O. rudis is regarded as the most important vector in the tropics in this hemisphere. The traditional vector, and the most common in West Africa, is O. moubata. There appears to be no specific relationship between any particular species of tick and any particular strain of spirochete.

Infection may persist for long periods in the tick; Francis has reported survival in starved ticks for as long as five years, and in re-fed ticks for six and one-half years. In addition, the infection persists in the insect vector by transovarial transmission, under experimental circumstances to the fifth generation.⁴¹

The spirochetes are present in the coxal fluid, saliva, and feces of infected ticks. Of these, the first two appear to be the more important in transmission of the infection to man. For example, O. moubata, an excellent vector, secretes coxal fluid copiously while feeding, thus making possible infection of the bite, while O. hermsi, which is not known to transmit the infection to man though it does so from rat to rat, does not pass coxal fluid while feeding. The relative importance of direct introduction of the spirochete into the bite by infected saliva is not altogether clear. It is probable that infection of the bite by contaminated feces occurs occasionally.

European relapsing fever is transmitted from man to man by the human body louse, Pediculus vestimenti, and has the epidemiological characteristics of louseborne dis-

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ease. In contrast to the tickborne infection, the bite is not infected by the secretions of the lice; rather the infected louse must be crushed on the skin and the spirochetes present in the body fluids contaminate the bite. The infection is transmitted to the egg, and there is some evidence that the disease may be produced by crushed eggs laid by infected lice.

It is possible that the fingers may become contaminated by crushing infected lice and transmit the infection by scratching. The bedbug may also serve as a mechanical carrier of the infection.

Spirochetosis of fowls is transmitted by a tick, *Argas persicus*, which infects fowls in the warmer parts of the world, and, as in human relapsing fever, the spirochete is transmitted through the egg of the tick to the offspring.

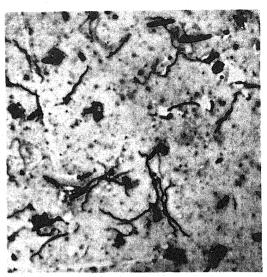


Figure 223. Smear from Vincent's angina showing the characteristic mixture of spirochetes (*Borrelia vincentii*) and fusiform bacilli. Fuchsin; × 1250. (P. E. Harrison.)

SPIROCHETES OF THE MOUTH

As described elsewhere (Chap. Twentyseven), spirochetes are a part of the normal flora of the mouth which, together with other microorganisms, may increase in numbers in ulcerative conditions of the mucous membranes and in peridontal disease. These forms are usually regarded as species of Treponema, viz., T. micordentium, etc. In the ulceromembranous stomatitis and angina known as Vincent's disease, Vincent's angina, or trench mouth, these forms. together with fusiform bacilli of the Bacteroides group, increase in numbers and predominate in stained smears from the necrotic lesion. Other bacteria, cocci, and bacilli are present also, and the typical microscopic picture is illustrated in the accompanying figure.

The spirochete found in such preparations is known as *B. vincentii* or *T. vincentii*, for the two genera cannot be separated on morphological grounds alone (see below). Whether this microorganism constitutes a distinct species of spirochete and plays any part in the development of the pathological process is questionable. It seems clear that the oral spirochetes are not pathogenic in the sense of being specific etiological agents of disease but, together with other microorganisms, may act synergistically in the accentuation, if not the initiation, of such suppurative infections.¹³

Treponema and the Treponematoses 47, 110, 117

Spirochetes of the genus Treponema are morphologically indistinguishable from Borrelia, but the pathogenic forms are set apart from the relapsing fever spirochetes by the kinds of diseases which they cause. The pathogenic forms are Treponema pallidum, the causative agent of syphilis; T. pertenue, the causative agent of yaws; T. carateum, the causative agent of pinta; and Treponema cuniculi, the causative agent of rabbit syphilis. T. microdentium and T. macrodentium are nonpathogenic, and a

part of the normal oral flora as indicated above, but are, in combination with fusiform bacilli, associated with ulcerative disease of the mouth, and this combination of microorganisms has also been found in certain types of tropical ulcer.¹⁰⁸

The pathogenic species of Treponema make up a group of interrelated microorganisms, separable by differences in the kinds of disease they produce. Conversely, the infections with these microorganisms, known collectively as the treponematoses,

are at the same time both similar, as with respect to the immune response, and differentiable by the character of the clinical disease. The treponematoses fall into three, or four, groups, viz., syphilis which is divided into the venereal and nonvenereal forms, yaws or tropical syphilis, and pinta or pintid yaws. The mode of transmission is thought by some to relate to differences in the diseases, i.e., the lack of systemic cardiovascular and neurological involvement in the nonvenereal forms.

The origin of treponemal infection has been a matter of absorbing and perennial interest. It is believed by some that an ancestral type of treponema, subjected to varying climatic, social, ethnological, economic, and other factors, may have differentiated into the several present forms of the microorganism, differing in their mode of transmission and in their pathogenicity with consequent variability in the human disease. 25, 51, 59, 60, 116

A disease similar to syphilis was present on the China coast in ancient times, the Biblical plague of Moab is thought to be syphilis, and syphilitic infection apparently occurred in Eastern Europe prior to the fifteenth century. Venereal syphilis, or morbus gallicus,58 occurred in epidemic form in Europe after the return of Columbus from the Americas, and it is believed by some that this form of treponematosis was introduced from the Western Hemisphere. There is evidence that syphilitic infection may persist in nonvenereal form following epidemics of venereal syphilis. The Scottish vaws or "sibbens" occurring in the seventeenth century after the time of Cromwell,70,82 "radesyge" in Norway, and "spirocolon" in Greece and Russia in the nineteenth century appear to be nonvenereal syphilis derived in this way. With improving economic and sociological conditions, the disease tends to die out-the last case of sibbens, for example, was reported more than 100 years ago – but it persists at the present time in the Near East, in Africa, and in Central Europe, and occurred in epidemic form in Chicago in 1949.

The earliest description of a yaws-like disease appeared in 1558 and occurred in Brazil, and the disease as known today was identified in the seventeenth century in the West Indies and in Brazil in the eighteenth century; it is now known to be ubiquitous in tropical regions. Pinta is more recently identified as a treponematosis occurring in

Mexico, Cuba, and adjacent areas; the spirochetal etiology was established in 1938 by Cuban workers on the basis of earlier studies by Herrejon; formerly it had been thought to be a fungous infection.

While the differences among these diseases are felt by some to require considering them as separate entities, the differences are not much greater than those between venereal syphilis in the Middle Ages and present-day venereal syphilis. It is believed by many that the similarities are more striking than the differences and these, together with other considerations, have led to a "unitarian" view of the treponemal infections in which the several clinical manifestations are considered to be variations on a central theme.

Cultivation. Schereschewsky reported the cultivation of T. pallidum in 1909, but his cultures appear to have been contaminated with other microorganisms. Noguchi later reported the growth of several strains of Treponema in pure culture under strictly anaerobic conditions in a serum water medium containing a piece of sterile fresh rabbit kidney or testicle. A number of such strains of spirochetes have been similarly isolated and may be grown in a heart infusion broth containing glycose, cysteine, and filtrate of coagulated plasma, the best known of which are the Reiter, Nichols, Kroo, and Kazan strains. In other instances the spirochetes may survive for extended periods, 113 but are not actually cultivated. The Reiter strain. although useful in serological studies, was originally virulent for rabbits when isolated by Wassermann and Ficker in 1922, later became completely avirulent but seems to have retained its antigenicity. The Nichols strain has retained virulence not only for the rabbit but also for man as shown by inoculation of human volunteers.73 In that study 57 spirochetes infected half of eight nonsyphilitic individuals, and the ID₅₀ for the rabbit was 23 spirochetes.

The Treponemas are extremely fragile microorganisms and die out quickly outside the body. They are destroyed by soap and water or by drying, and are unusually susceptible to heat; suspensions of infected rabbit testicle are sterilized at 41.5° C. in one hour and in two hours at 41° C., but the cultivable strains may remain viable in culture mediums kept at 37° C. for as long as a year. The viability of T. pallidum is of interest in connection with the possibility of transmission of syphilis by infected blood;

although occasional transmission has occurred in this way, the risk is not great with blood from blood banks, for the spirochete dies out in three or four days at refrigerator temperature and quite rapidly in lyophilized plasma.

Pathogenicity for animals. Syphilis may be transmitted to anthropoid apes, such as the chimpanzee and gibbon, and, with less certainty, to monkeys, as shown by Metchnikoff and Roux in 1903. Scarification of the genitals or eyebrows results in development of a primary chancre followed in a few weeks by the appearance of lesions of secondary syphilis.

Rabbits may be infected by inoculation of the anterior chamber of the eye. The local wound heals, but in four to six weeks pericorneal congestion develops which is followed by pannus and keratitis; then retrogression and healing occur. The entire process may take many weeks to complete. Intratesticular inoculation or implantation produces orchitis and intrascrotal inoculation a primary chancre; generalized lesions characteristic of secondary syphilis follow. A generalized infection may be produced by intravenous inoculation of very young rabbits. The spirochetes remain alive indefinitely in the lymphatics and may be obtained by excision of a popliteal gland. In rats and mice the infection is symptomless, but the spirochetes multiply in the tissues and the infection remains latent. Guinea pigs react similarly but may develop a local reaction following intracutaneous inoculation in the perineal fold.

The rabbit has been the experimental animal of choice, and the reaction to intradermal inoculation has allowed the differentiation of strains of Treponema with respect to pathogenicity. A small inoculum suffices to infect, and multiplication occurs exponentially after 24 to 48 hours, with the production of a mass of microorganisms and a visible lesion. With an inoculum of 500 spirochetes the incubation period is about 17 days; with an inoculum of 5000 microorganisms, four to five days, and the generation time is estimated to be about 30 hours.

In the initial stage hyaluronic acid is produced which appears to favor the multiplication of the microorganisms. In the second stage there is a mononuclear infiltration, and in the third stage an influx of polymorphonuclear leucocytes, and the infection may or may not involve the regional lymphatics.

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Three kinds of reaction in the rabbit, and also in the hamster, have been described by Turner and Hollander. 110 One of these, designated the Sh or syphilis type of reaction and given by T. pallidum from venereal syphilis, consists of an indurated lesion at the site of inoculation and lymphatic involvement. Samoan strains of T. pertenue produced only minimal local reaction and no infection of the lymphatics, designated the Yh reaction. An intermediate reaction, the Mh type, with pronounced local lesion and lymphatic infection, is given by some yaws strains and by spirochete strains from nonvenereal or endemic syphilis. Although it has been reported⁶⁹ that the rabbit may be infected with T. carateum, the infection has not been fitted into these types.

Immunity. The immune response in treponematosis is two-fold. The antibody-like activity designated reagin has wide diagnostic utility (see below), characteristically reacts in complement fixation and flocculation tests with nonspecific cardiolipid prepared from normal tissues, and its titer is correlated with the status of the clinical disease, disappearing when cure by chemotherapy is effected. The other is an antibody response to antigens present in the spirochetes, demonstrable with spirochetal antigens as immobilization and other phenomena (see below). It is generally believed that there are three kinds of antigens present in the spirochetes, a thermolabile protein antigen, a thermostable polysaccharide antigen, and a lipoidal antigen similar to the material present in cardiolipin preparations. There appears, however, to be no immunological difference between the spirochetes of venereal syphilis, nonvenereal syphilis, yaws, and pinta, and all of these diseases give positive reactions to the same serodiagnostic tests. It has been reported³⁴ that T. pallidum shares a common antigen, the Reiter protein antigen, with the Reiter treponeme, T. microdentium, and T. zuelzerae, but by the use of absorption or blocking procedures and application of fluorescent antibody methods, they may be differentiated from one another.

TREPONEMA PALLIDUM (Syphilis)

Syphilis, by far the most intensively studied of the treponematoses, occurs, as

indicated above, in two forms. One is venereal syphilis and is the best known form, and the other is nonvenereal or endemic syphilis. The two are epidemiologically distinct in that transmission is almost always sexual in the first instance, but in the second the infection is transmitted both by direct nonsexual contact and indirectly by the common use of eating and drinking utensils among children and adolescents. Consistent with this, the primary lesion is usually on the genitalia in venereal syphilis, but the first lesion occurs as oral mucous patches in the childhood disease.

The prevalence of syphilis is not known definitely; the estimate that 10 per cent of adults in the United States will give positive Wassermann tests is probably fairly accurate. It is also estimated that between 1 and 2 per cent of the children in this country have congenital syphilis. The mortality from syphilis is also not known. The death rate of 13 to 14 per 100,000 is misleading, for deaths reported as due to other causes are frequently due to syphilis; all cases of general paresis, locomotor ataxia, and many cases of apoplexy and aortic aneurysm are, for example, due to syphilitic infection, and the disease is a contributory cause in deaths from other diseases. Studies on the incidence of syphilis in autopsies on adults have given rates of from 2.6 per cent to 29.5 per cent; the average in 146,761 autopsies performed from 1896 to 1938 was 5.45 per cent. In this

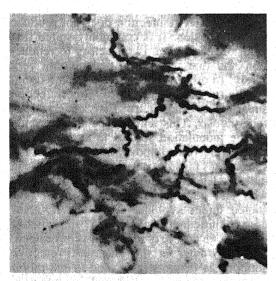


Figure 224. Treponema pallidum in stained (silver impregnation) smear. × 3000. (Kral.)

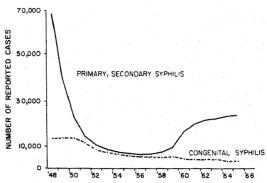


Figure 225. The prevalence of syphilis as indicated by reported civilian cases in the United States during the period 1948–1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare.)

country the prevalence is much higher in the Negro than in the white; tests of the first two million selectees under the Selective Service Act showed 2 per cent in whites and 22 per cent in Negroes. There is considerable variation in the ratio from one part of the country to another.

The number of reported cases continued to fall in this country until 1955, remained steady until 1958, then increased from 113,894 in that year to a peak of 126,245 in 1962, and has since declined to 112,842 in 1965. Such data are only relative, for many cases of syphilis are not reported. It is estimated that the reservoir of untreated syphilis cases is 1,200,000, and that the annual increase is about 60,000. There are currently about 33,000 paretics in institutions. ¹⁸

The disease in man. A disease of protean manifestations, syphilis in man assumes several relatively well-defined clinical stages. The ordinary course of the disease includes the successive appearance of the so-called primary, secondary, and tertiary stages; congenital syphilis is best regarded as a separate subdivision.

Primary stage. Following the penetration of the skin or mucous membranes, the spirochetes rapidly invade the tissues; it has been found that the inguinal lymphatics of rabbits become infective 30 minutes after cutaneous inoculation of the scrotum. The initial primary lesion appears 10 to 30 days following infection. The typical hunterian chancre (so-called because it was described accurately and in detail by John Hunter) is an indolent, indurated ulcer, single and painless, and not

infrequently atypical and trifling in appearance. The presence of the spirochetes in the chancre serum may be demonstrated by darkflield examination and constitutes the diagnostic method in early primary syphilis, for a positive Wassermann reaction (see below) does not develop until two or three weeks after infection. Clinically, then, syphilis in the primary stage is a localized infection, but bacteriologically it is a generalized infection, and spirochetes are often present in the blood stream.

Secondary stage. In the secondary stage the generalized nature of the infection is apparent. Bones, joints, eyes, and other organs are invaded, with the appearance of constitutional symptoms, cutaneous lesions, enlargement of the lymph glands, pains in the joints, and the like. Evidences of the development of this secondary stage are usually apparent four to eight weeks after the appearance of the chancre but may be delayed for a year or more. Spirochetes are present in all the lesions and may be found in the blood stream.

Tertiary stage. The disease passes insensibly into the tertiary stage and may persist for years. Ulcerating necrotic lesions of the skin and gummata of the internal organs are common, and the clinical manifestations of infection are so highly diverse that there is no characteristic picture. Spirochetes are present in the tertiary lesions but only in small numbers.

Neurosyphilis. Spirochetal invasions of the central nervous system may occur independently of the so-called stages and as soon as the early secondary stage. Generally, however, neurosyphilis is a manifestation of late tertiary infection and may appear years after the initial infection, and tabes dorsalis, general paresis, and other evidences of the invasion are sometimes termed quaternary or parasyphilitic affections. The spirochetes may be found in the cord in tabes dorsalis and in syphilitic brains in the cortex, in the pia and vessel sheaths. General paresis is, then, a diffuse spirochetosis of the brain involving the cortical layers chiefly, and the presence of the spirochetes in the parenchyma explains much of the histopathological picture and the nervous symptoms of the affection.

Latent syphilis.²⁸ In some instances the primary lesion is not noticed and the secondary manifestations may be slight, and with their subsidence there is no clinical evidence

of disease and the infection remains latent within the tissues. In other instances the latent condition develops after primary and secondary symptoms of usual severity. Such clinically latent infections may, perhaps, be regarded as the maintenance of a biological balance between the host and the parasite. Latent infections may, of course, develop clinical manifestations at a later time.

Congenital syphilis. The fetus is readily infected in utero by spirochetes passing through the placenta. The infection may result in a cessation of development and abortion, or the fetus may go to term and be born dead. In those who survive, the disease is generalized from the beginning, and the lesions are those of tertiary syphilis. Spirochetes may be demonstrated in great numbers in the tissues and organs affected. Third generation syphilis has been reported but is very rare. 93

Immunity. 19, 115 At the present time syphilis is not so severe a disease in man as it was in the early sixteenth centry. Whether this is an expression of an adaptive response on the part of man with the development of a low degree of natural immunity, or a similar response on the part of the parasite manifested as a decrease in virulence, is not known; possibly both may have occurred.

The specific immune response to syphilitic infection is not well understood. There is a marked apparent insusceptibility to reinfection that is illustrated by the fact that a second chancre may be produced by reinfection prior to the appearance of the first chancre, but after the first chancre has appeared further reinfection does not produce an initial lesion. It is commonly stated that man, once infected, is refractory to reinfection and that reinfection occurs only very rarely. In other words, there is an infectionimmunity or immunity to superinfection superficially similar to that observed in tuberculosis. This tends to be substantiated by the inoculation studies in human volunteers⁷³ referred to above, in which 100,000 spirochetes failed to infect individuals having untreated latent syphilis, 10 of 26 persons who had been treated for proved or presumed latent syphilis, while the ID₅₀ for nonsyphilitic individuals was 57 spirochetes.

A certain degree of immunity is developed, especially later in the disease. Protective antibody is demonstrable, and in the rabbit a partial immunity, produced by infection and cure with penicillin after varying times,

inhibits to a certain extent dissemination of infection following subsequent inoculation. In addition, the spirochetes are immobilized and killed in syphilitic serum in the presence of complement. An *in vitro* immobilization test has been devised for the demonstration of this activity (see below), but it is not clear to what extent it is associated with effective immunity.

Serodiagnosis.¹⁰⁴ As indicated above, the immune response is of two kinds, the appearance of Wassermann antibody, or reagin, and of antibody to antigenic substances present in the spirochete. The former has been widely used for diagnostic purposes, while the latter response is immunologically specific, but its assay is as yet too complex for general application.

A complement fixation test was proposed by Wassermann and his co-workers in 1906 as an immunologically specific reaction, using a watery extract of syphilitic fetus as the antigen. It was soon found, however, that syphilitic serums fix complement in the presence of an extract of normal tissue.

Not long after the development of the Wassermann test it was observed that syphilitic serums produce a flocculation when mixed with Wassermann antigen. The reacting substance present in the antigen preparations occurs in various tissues, but heart (beef) muscle extracts are the most satisfactory. It is soluble in alcohol and insoluble in acetone, and its activity is considerably increased by the addition of cholesterol. Activity of these extracts is due to a phospholipid, cardiolipin, which is precipitated from the initial alcoholic extract with cadmium chloride, freed from lecithin, and fractionated by precipitation from ether with acetone and alcohol. Anticomplementary activity of the preparation is removed by the addition of lecithin,68 and complement is not fixed unless cholesterol is added. The function of lecithin is not clear, and it may function only as an emulsifying agent. The complete lecithin structure seems to be required, and dimvristovl lecithin may be substituted for natural lecithin.3 According to Pangborn, 80 who described this substance in 1941, it is a complex phosphatidic acid. A similar product of vegetable origin (wheat germ) sitolipin, has been prepared. The activity appears to be a property of similar substances varying in their fatty acids; lauric and linoleic acids have been found to be present, together with a third component.90

Types of tests. Probably no serological reactions have been as intensively studied as the complement fixation and precipitation tests for syphilis, and the techniques have been modified and refined by many workers to increase sensitivity and specificity. At the present time two complement fixation tests, the Kolmer test and the Venereal Disease Research Laboratory (VDRL) test, and four precipitation tests, the Kahn test, the Kline test, the Hinton test, and the Eagle precipitation test, are used. In addition to these. more sensitive precipitation tests have been devised by Kahn and by Kline and are known as the Kahn presumptive test and the Kline exclusion test: they are apt to give false positive reactions but are regarded by some workers as having considerable utility as screening tests. A rapid unheated serum reagin, USR, is used also for presumptive purposes.114

The complement fixation test may also be quantitative; *i.e.*, the reagin present in the syphilitic serum may be titrated. For ordinary diagnosis this is unimportant since it is not known whether the reagin titer has any significance, but it is useful in following therapy and in the diagnosis of congenital syphilis in infants. In the latter case reagin is passively transferred from the maternal circulation; if the infant is not infected the reagin titer drops slowly, but a gradually increasing titer is indicative of infection.

Specific tests. Any serological test making use of spirochetal antigen, and thus having immunological specificity in the conventional sense, should be much more precise. An antigen may be prepared from T. pallidum by making an extract of infected rabbit testicular tissue with citrate, acetone, and deoxycholate for use in the T. pallidum complement-fixation, or TPCF, test.⁷² The antigen may also be obtained by differential centrifugation of T. pallidum from infected rabbit tissue, cryolysis, and ammonium sulfate precipitation to give the T. pallidum cryolysis complement fixation, or TPCP, test. The cultivable Reiter strain contains antigens in common with T. pallidum as demonstrable by absorption tests and may also be used as a source of antigen in the Kolmer-Reiter protein complement fixation. or KRP, test.44

The treponema immobilization test (TPI), in which the living motile spirochetes are specifically immobilized in the presence of antibody, complement, and thioglycollate

(the last to maintain anaerobic conditions), has been devised. It has been widely and critically tested and found to be highly specific. 16 As originally carried out, it required 18 hours, but it has been found that the addition of egg white lysozyme reduces the required time to six hours. 77 The fluorescent antibody technique has also been applied to the serodiagnosis of syphilis, using both the direct and indirect methods 32, 33 to give a fluorescent treponemal antibody, or FTA, test. The test is highly sensitive and correlates well with other serological methods. 39, 79

The wide variety of serodiagnostic tests is an expression of a continuing search for a completely reliable test, but it is probable that no single test will suffice in the face of complications of therapy, biological false positives and negatives, etc. Some combination of tests may be required as indicated by circumstances (see below); one such combination which has been suggested²⁰ is that of the VDRL slide test, the RPCF test, and the TPI test.

The effect of therapy. The majority of individuals undergoing therapy become serologically negative to the cardiolipin type of test in time, and this response is usually taken as an indication of the efficacy of the treatment. The time required to attain negativity is variable and depends upon the individual, the stage of the disease, the course of therapy, especially if continuous or intermittent, and the sensitivity of the serological test used. No general statement of any precision is possible, therefore, but it is usually said that the majority of persons with primary or early secondary syphilis become negative after one or two courses of arsenical or continuous treatment for five to seven weeks. Some, however, become negative with only one or two injections of drug, while others may remain positive for months, and a certain proportion remain positive indefinitely. The last is spoken of as Wassermannfast, reagin-fast, or seroresistant syphilis.55 Slightly over 10 per cent of infections are in this group. Two explanations are usually offered for seroresistant syphilis, one that persistent foci of infection remain, and the other that the continued presence of reagin is evidence of a definite immune response.

Specificity and false reactions. False negative reactions are in part a technical matter; serological tests in general use give 80 to 90 per cent positive reactions with known serums, and any test which gives more than

80 per cent positives is regarded as satisfactory. The presence of insufficient reagin accounts for many false negative reactions and occurs in several stages of the disease. especially in early primary syphilis (a positive reaction usually does not develop until two to three weeks after infection) and late syphilis which is latent or localized. Tabes, for example, gives about 40 per cent negative reactions. Negative reactions may also be a result of the presence of antibody to the Forssman antigen, acquired for example by prior infection with microorganisms containing the antigen, such as certain strains of pneumococci, in the serum tested. If such antibody is present to reasonably high titer. the sheep cells of the indicator system are lysed in the presence of complement. This may be avoided by a preliminary absorption of the serum with sheep cells.

False positive reactions may be a result of technical error or may be biological in nature. A certain proportion of nonsyphilitic individuals suffering from other infections give positive serological tests for syphilis. All cases of yaws are Wassermann-positive, and it is reported that 4 to 10 per cent of cases of malaria are positive. Brucella infection has also been reported²¹ to be associated with false positive reactions. Febrile disease of one sort or another occasionally produces isolated or repeated positive reactions. Biological false positive reactions to the cardiolipin tests are thought to be often associated with collagen disease.⁶³

False reactions occur less commonly with the specific tests using treponema or treponemal antigen, and these have their greatest utility when the more readily performed slide precipitation and other kinds of nonspecific reactions give equivocal results.^{24, 40} The treponemal tests are subject to error also, but these may be technical in nature.⁹⁴

The nature of the response to infection with the spirochete that results in the appearance of reagin is not clear. Et lack of immunological specificity would seem to rule out the usual type of antibody response, but if it is to be regarded as antibody, the facile disappearance of reagin during therapy rather than seroresistant syphilis requires explanation. It is possible that reagin represents iso-antibody that is detectable only because it is being produced more rapidly than it can be removed by union with antigen.

Chemotherapy. The chemotherapy of syphilis, like that of tuberculosis, has been

of great interest for many years. The long-term treatment with arsenicals and bismuth was highly effective for the relatively small number of patients who did not default. For example, of 1340 cases treated over a period of four years and followed for an average of 11 years, there were only nine failures of which four were reinfections. This method of treatment is now a thing of the past although arsenicals and/or bismuth have been used in combination with penicillin. 23, 61

Of the chemotherapeutic drugs now available, the sulfonamides are ineffective, but penicillin has been found to be highly effective. For example, in the research series of the U. S. Public Health Service, 1538 patients treated on various schedules were followed for two years. In this group 92.3 per cent of those having intially seronegative primary syphilis, 82.4 per cent of those with seropositive primary syphilis, and 78.3 per cent of those with secondary syphilis were cured. The relative efficiency of penicillin therapy and the adjuncts made use of are dependent upon the stage and nature of the disease.²⁹

The broad-spectrum antibiotics, chloramphenicol, and the tetracyclines are apparently almost as effective as penicillin in the chemotherapy of syphilis. As yet only relatively small numbers of patients have been treated with them, and their ultimate value can be determined only by studies carried out with large numbers of patients over a period of years. While as yet the incidence of penicillin-resistant spirochetes has not assumed practical importance, the occasional occurrence of penicillin-resistant strains coupled with individual sensitivity to this drug is indicative of the utility of alternative chemotherapeutic agents.

NONVENEREAL SYPHILIS

Nonvenereal or endemic syphilis occurs in foci in many parts of the world under various local names. That occurring among the desert Arabs in the Near East is known as bejel,⁵⁷ that in Karanga people in southern Rhodesia is njovera, dichuchwa is the disease found among the Bantus in Bechuanaland,⁷⁶ and the disease is known locally as siti in Gambia. It occurs in southeast Asia and with some frequency in eastern Europe, especially in Bosnia in Yugoslavia.⁴⁵ In the last area, prior to mass treatment under WHO auspices, patients with the disease

made up as much as 5 per cent of the population.

The disease is an epidemiological rather than clinical entity, occurring, as indicated earlier, largely in children and within families. There appears to be little or no basis for separating the diseases occurring in widely separated foci from one another or from venereal syphilis. Clinical differences between venereal and nonvenereal syphilis, viz., the relative rarity of primary lesions and congenital manifestations in the latter, seem to be a consequence of the mode of transmission and age distribution of the disease rather than of differences in the pathogenicity of the causative microorganism. There is, however, some uncertainty as to the frequency of cardiovascular and neurological involvement; it seems to be relatively rare, but not unknown, in nonvenereal syphilis, but the nonvenereal form of the disease may occur mixed with venereal syphilis.

The first manifestations of the infection are most commonly mucous patches in the mouth, and skin lesions including anogenital condylomata occur also. These lesions show the presence of spirochetes on darkfield examination, as do the lesions found on the nipples of mothers infected by children. In most individuals latency occurs after the early stages, with subsequent development of tegumentary lesions, including gummatous destruction of the skeleton, which are indistinguishable from those of venereal syphilis. The causative microorganism is *T. pallidum*, and the disease is effectively treated with repository penicillin.

TREPONEMA PERTENUE (Yaws)87, 103

Yaws, or frambesia tropica, is a disease occurring in tropical countries and is common in Equatorial Africa, the tropical regions of the Far East though rare in India, the West Indies, and tropical America. The incidence may be high, 5 to 20 per cent, and the disease is a major public health problem in Haiti. It is prevalent in rural rather than urban areas and associated with low economic status, i.e., the wearing of clothing appears to protect. Primary infection is more common in children than in adults. The causative agent is T. pertenue, which was described by Castellani in 1905.

The spirochete is found in the serous ex-

udate of the cutaneous lesions and in the lymphatics. It may be demonstrated in Giemsa-stained smears or by darkfield examination of fresh preparations and is morphologically indistinguishable from *T. pallidum*.

The disease in man49 is characterized by a papular eruption. The commonest site of infection, perhaps in 75 per cent, is the lower leg and foot. A general malaise precedes the appearance of the initial lesion, which is practically always extragenital and takes the form of a single papule or a small group of them. The papule enlarges to a diameter of 3 to 4 mm., the thickened epidermis cracks, and the fungoid mass beneath exudes a seropurulent fluid. The mass enlarges to 3 to 5 cm. in diameter and the lesion is termed a yaw. When the infection occurs on the sole of the foot, it is known as crab vaws or wet crab yaws. It eventually dries, leaving only a scar. Six weeks to three months after the primary lesion appears, a secondary eruption occurs which is similarly preceded by a general malaise. The lesions are of the same general character as the primary lesion, appearing on the extremities, neck, and at the juncture of the skin and mucous membrane about the nose, mouth, and anus. Tertiary lesions similar to those of syphilis are said to be rare, but recent work suggests that they may be more common than has been supposed. The Wassermann reaction is positive, and in certain stages, as in late secondary eruption, differential diagnosis may be very difficult.⁵²

Yaws may be produced by inoculation in monkeys and rabbits. The infection in rabbits with *T. pertenue* is similar to that with *T. pallidum*, but relatively few spirochetes are found in the lesions, multiplication is arrested early, and the inflammatory reaction is slight.

The disease is seldom venereal, and the commonest mode of transmission is contact between individuals.^{48,50} Trauma of the skin usually precedes infection, and the ability of the spirochete to penetrate the intact skin is questionable. Biting flies may also transmit the disease, and in Jamaica the small fly, *Hippelates pallipes*, feeds upon the serous exudate in huge numbers, the

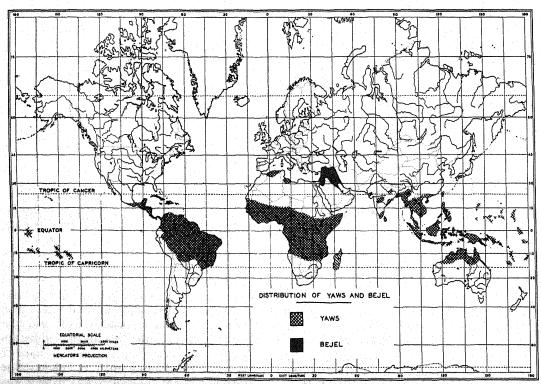


Figure 226. The world-wide distribution of yaws and bejel. (Redrawn from map prepared by Army Medical Intelligence. Based on Goode Base Map No. 201M. By permission of the University of Chicago Press.)

spirochete surviving for some hours in the diverticulum of the fly and possibly being regurgitated when the fly feeds upon a skin abrasion.

Yaws may be successfully treated with penicillin, and there is evidence that chloramphenical and tetracyclines may also be effective. The change to seronegativity is usually not as rapid as in effectively treated syphilis, possibly because the disease is usually of longer standing.

The relationship of yaws to syphilis is very close, the points of difference being the nonvenereal transmission of yaws, the differences between the yaw and the indurated hunterian chancre, and the relative infrequency of visceral and tertiary lesions. Yaws is regarded by many as a tropical type of syphilis, differing, not necessarily qualitatively, from endemic syphilis as a result of environmental factors. There is some evidence of a cross-immunity between syphilis and yaws in that the two diseases tend to be mutually exclusive, but precise evidence is lacking.

TREPONEMA CARATEUM (Pinta)^{22, 56, 74}

Pinta, known in Mexico as mal de pinto and in Colombia as carate, is clinically a skin disease in which dyschromic changes occur in patches which may be discrete and small or large and confluent, and of a gray, bluish gray, or pinkish color and eventually white. It is especially prevalent in Mexico, where 11 per cent of over two million persons examined had the disease, and in Colombia, where as much as 4 per cent of the population in some districts are affected. It also occurs in Cuba and the West Indian Islands and in Central America and tropical South America. There is some question as to whether the Mexican and Cuban diseases are identical.¹¹¹ It is apparently confined to the Western Hemisphere for the most part, but cases have been reported from India.42

Etiology. For many years this disease has been of uncertain etiology though generally assumed to be a fungous infection. It was suggested by Herrejon in 1927 that the disease is caused by a spirochete, but it was not until 1938 that a spirochete was first demonstrated by Armenteros and Triana in Cuba. The observation has been amply con-

firmed, and it is now established that pinta is caused by a spirochete which has been named *T. herrejoni* by the Cuban workers and *T. carateum* by Brumpt.

The spirochete may be found in material taken from early cutaneous lesions and in that aspirated from lymph glands. It stains with Giemsa and by the usual silver impregnation methods used for T. pallidum and may be found by darkfield examination of fresh material. Morphologically it is very similar to, perhaps indistinguishable from, the spirochete of syphilis. While it gives no cross-reaction with T. pallidum in the immobilization test, the pattern of the universal serological reaction of Kahn is identical with that of syphilis. Attempts to cultivate it have failed, but it has been reported that the rabbit may be infected. The experimental disease in human volunteers has been studied in considerable detail, however, especially by Leon y Blanco.

The disease in man. In the experimental infection the incubation period is from seven to 20 days, with the initial lesion being a papule which appears at the site of inoculation. This papule spreads peripherally to form a squamous, erythematous patch reaching a diameter of 1 cm. in four or five weeks. It continues to spread, varying considerably in appearance, and may be lichenoid or psoriaform. There is less inflammatory reaction than in either syphilis or yaws.⁵³ Leon regards this as the primary stage. In about five months the secondary stage begins with the appearance of secondary lesions about the initial lesions and elsewhere on the body. Progressive hyperpigmentation occurs, and depigmentation, resulting in varied hues, follows in the third stage; keratosis and superficial atrophoderma become apparent. Hyperkeratosis on the plantar surfaces occurs in Cuba but is seldom observed in Mexico. The Wassermann reaction is almost always negative in the primary stage, positive in something over half the cases in the secondary stage, and practically always positive in the tertiary stage. Correspondingly, superinfection is readily produced in the first stage but not in the third stage. Syphilitic individuals may be infected without difficulty. Pinta may be successfully treated with penicillin.

Transmission. 98 Experimental human infections have shown that serous fluid from the early lesion is highly infectious when placed on an abraded area on the skin, and

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the spirochete may be demonstrated in the discharge from the fissures in plantar hyperkeratosis. Contact infection would appear to be possible, but general opinion is that it is not an important method of spread. Simulium flies have been suspected of transmitting the disease, but as yet an insect vector has not been demonstrated.

Leptospira and Leptospirosis 4, 5, 105

Leptospirosis, or infection with leptospira, is a not uncommon and widely distributed affection. Several species of leptospira are involved, and the infections differ somewhat in their clinical manifestations.

Morphology. The leptospiras differ from Treponema and Borrelia species in that their spirals are very fine and close, one or both ends of the cell may be hooked, and the individual cells are smaller, not more than 0.3 μ in breadth and generally 6 to 10 μ in length, although exceptionally forms as long as 25 to 30 μ may be observed. The protoplasm is spirally wound about a delicate but firm. elastic, smooth axial filament enclosed within a well-defined cell wall, which is clearly shown in electron micrographs. 91, 97 Like the finer bacterial flagella, the axial filament is not seen in the darkfield or ordinary stained preparations but may be demonstrated by silver impregnation flagella stains. The

periplast is thick and almost transparent and appears in the darkfield as a narrow, clear zone or halo and in certain stained preparations as a grayish or unstained halo. The mechanism of locomotion is more complex than in the treponemas; the hooked ends of the axial filament are probably involved, and when the bow-like axial filament is alternately straightened and relaxed by rhythmic contractions of the protoplasmic spirals the cell rotates. Their small breadth and active motility allows penetration of filters such as the Berkefeld V and N candles, and filtration is often used on primary isolation from contaminated materials to separate them from other bacteria.

Cultivation. 66 The leptospiras are the most readily cultivable of the spirochetes. They may be grown in a liquid medium consisting of 1 per cent peptone in phosphate-buffered Ringer solution to which sterile

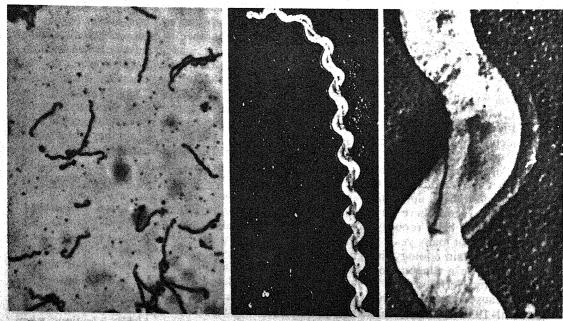


Figure 227. Leptospira morphology. Left, conventional stained smear of Lept. pomona. Center and right, electron micrographs of chromium-shadowed spray preparations of Lept. icterohemorrhagiae. Center, × 12,500; right, × 125,000. (Simpson and White. **)

LEPTOSPIRA 761

rabbit serum is added in a final dilution of 1:40, or in a semisolid medium consisting of meat extract agar enriched by the addition of sterile serum. In the isolation of leptospira from the blood, the inoculum is in the proportion of 0.03 ml. of blood to 15 ml. of medium, and the culture is incubated for as long as 28 days before discarding. The blood presumably satisfies the nutritive requirement for iron. One of the most readily prepared liquid mediums for the cultivation of leptospiras is that described by Cox.26 consisting of 0.2 per cent tryptose-phosphate broth enriched by the addition of normal rabbit serum to 10 per cent. Some strains have been grown in chemically defined mediums. 100, 112 In the isolation of leptospira from water, agar medium and blood may be added to Berkefeld V and N filtrates. The leptospiras are all obligate aerobes. Those tested do not ferment sugars and are indifferent to the presence of carbohydrates in the medium.

Colonial growth of leptospiras on solid medium has been described by Cox and Larson²⁷ on tryptose-phosphate-normal rabbit serum medium enriched with rabbit hemoglobin and solidified with 1 per cent agar. Two types of colonies were observed, a small relatively opaque type and a larger translucent form ranging in size from 1 to 4 mm, in diameter.

Cultures are maintained in liquid or soft agar medium which may be covered with a thin layer of paraffin oil to retard evaporation. The cultures are incubated at 28° to 30° C. for one to two weeks, during which leptospiral growth appears as a band of turbidity a few mm. below the surface. Such cultures remain viable for five to six months when stored at room temperature, and should be transferred as 0.5 ml. of inoculum every three to four months. For serological purposes the cultures should be young, one to three weeks old. It has been reported that leptospira may remain viable when stored in dry form at refrigerator temperatures.

Antigenic structure. 10, 89 The leptospiras are excellent antigens, and agglutinating, lytic, and complement-fixing antibody is produced to high titer and persists in the case of recovered individuals, man or lower animal, to allow diagnosis of past infection. The several species or types of leptospira are differentiable and characterized on an immunological basis, and many serotypes have been established, with new serotypes being added from time to time.

The serotypes have been given place or descriptive names, implying that they are different species, but no formal classification within this group is firmly established. It has been tentatively suggested⁸⁸ that only two valid species be recognized, *Lept. interrogans*, including the parasitic forms, and *Lept. biflexa*, the saprophytic strains, with no taxonomic status for sertotypes or groups of serotypes. A collection of serotypes is maintained at Amsterdam in Europe and at the Walter Reed Army Medical Center in this country.

These serotypes appear to be quite stable although antigenic changes can be produced by cultivation in antiserum.⁸¹ There are minor cross-reactions with some other microorganisms, including Shigella strains.⁹² Immunity to experimental infection is largely type-specific with only occasional cross-protection between serotypes.

Pathogenicity. The pathogenic leptospiras are parasites of lower animals, including wild rodents and a variety of domestic animals of which the pig and dog, and in some areas cattle, are probably the most important quantitatively. Unlike Borrelia, they are not primarily blood parasites although they may be found in the blood as early as the first day and perhaps as late as the fourteenth day of the disease. The infection localizes in the tissues and organs, especially the kidneys, and the leptospiras are excreted in the urine.

Human infection is acquired from lower animals, by direct contact with infectious urine or water contaminated with infected urine or by direct contact with infected tissue. In the first instance the source of infection is most often rodents, and the leptospira penetrate the skin, and in the second, domestic animals, especially pigs, which serve as a source of infection for slaughterhouse workers, veterinarians, and herdsmen, as in swineherd's disease in Switzerland.

Leptospiral infections in man are all closely similar and are characterized by a febrile reaction, jaundice which may be generalized or confined to the sclera, and infection of the kidneys.^{7,8} Weil's disease, or infectious jaundice, is the classic example of leptospirosis and has been known for many years (see below).

Other infections with leptospiras are much more common than has been realized. In many instances these are acquired through contact with mice and other infected rodents, and, since the infection often occurs in connection with working in cultivated fields and similar rural areas, the disease is variously known as swamp fever, field fever, harvest fever, and the like. Such leptospirosis is given place names also, such as Mossman fever and Pomona fever, but in many, if not most, cases infection is much more widespread than this would suggest. The etiology of these diseases is nonspecific in that there is little specific association of a give disease with leptospiral serotype.

Recovery from the disease is accompanied

by the appearance of specific antibody and the development of an effective immunity. Prophylactic inoculation with leptospiral vaccines has been applied, with encouraging results, to groups of persons exposed to infection.⁹

Laboratory diagnosis. 86, 118 The laboratory diagnosis of leptospirosis is based upon isolation of the infecting microorganisms and on the immune response to the infection. In man the bacteria may be isolated from the

The More Important Leptospiroses*

SEROTYPE	DISEASE IN MAN	IMPORTANT ANIMAL RESERVOIRS	INFECTIONS IN OTHER ANIMALS	GEOGRAPHICAL DISTRIBUTION
icterochemorrhagiae	Weil's disease	R. norvegicus, other rodent species	Dog, pig, cattle, horse	World-wide
canicola	Canicola fever	Dog	Pig, cattle	World-wide
pomona	Pomona fever Swineherd's disease	Pig, cattle, Mus musculus, Apode- mus agrarius	Dog, horse, opos- sum, raccoon, skunk, wildcat	World-wide
grippotyphosa	Mud fever Schlammfieber Field fever	Microtus arvalis Evotomys glareolus Cricetus spp. Apodemus sylvaticus	Cattle, horse, dog, raccoon, goat	World-wide
autumnalis (akiyami A)	Hasami fever Fort Bragg fever	Microtus montebelloi Apodemus speciosus Bandicoota spp.	Dog, opossum, raccoon, cattle	S. E. Asia, Japan, U. S.
bataviae	Indonesian Weil's disease Rice-field fever	R. norvegicus Micromys minutus R. rattus	Dog, cat	S. E. Asia, Europe, Africa, Japan
australis A (ballico)	Canefield fever	R. conatus Apodemus flavicollis R. rattus culmorum	Dog, cattle, raccoon, opos- sum, hedgehog	Australia, U. S. Europe, S. E. Asia, Japan
australis B	Canefield fever	R. rattus, Isoodon spp.		Australia, S. E. Asia, Europe
sejroe	Feldfieber B	Mus musculus Apodemus sylvaticus Apodemus agrarius Microtus spp.	Cattle (?), dog, pig	Europe, U.S. (?
hebdomadis	Nanukayami Akiyami B Seven-day fever	Microtus montebelloi	Dog, cattle	Japan
pyrogenes	Leptospirosis febrilis	R. rattus R. brevicaudatus		S. E. Asia, Japan
ballum		Mus musculus, opossum	R. norvegicus, skunk, racoon, wildcat, pig	U. S., Europe Israel
hyos (mitis)	Swineherd's disease	Pig	Cattle	U. S., Europe, Australia, South America New Zealand

^{*}Compiled by Colonel M. B. Starnes, Walter Reed Army Medical Center.

blood early in the disease by culture or animal inoculation; for the latter, young (150 gm.) guinea pigs or weanling (15 gm.) hamsters are the usual animals. The experimental infection may be substantiated by demonstration of the leptospira in the tissues, especially in the kidney, on culture at autopsy, but in general animal inoculation gives a smaller proportion of positive results than does culture.

The immune response may be assayed as leptospiral agglutination (observed in the darkfield microscope), as lysis in the presence of complement, or by complement fixation. The agglutination-lysis titers range from 1:400 to 1:100,000, and the complement-fixation titers from 1:32 to 1:256. A four-fold or greater rise in antibody titer in paired serums is regarded as diagnostic, but in an appreciable portion of cases (10 per cent?) demonstrable antibody is not present. The immune response in infection is ordinarily of sufficiently broad specificity that the serological identity of the infecting microorganism is not deducible from it, and for precise identification the leptospira must be cultured and typed serologically.

Chemotherapy. Penicillin, tetracyclines, and erythromycin are somewhat effective in the early stages of experimental infections, and the last two, and streptomycin, may have some effect on the kidney infection. Chloramphenicol seems to have little or no value. In general, chemotherapy of human infections has been disappointing.

WEIL'S DISEASE (Infectious Jaundice)

The causative agent of Weil's disease, Leptospira icterohemorrhagiae, was discovered in 1914 by Inada and Ito in Japan and was found in Germany the following year by Hübener and Reiter and by Uhlenhuth and Fromme. It was called Spirochaeta icterogenes by the German workers. The disease has been found all over the world; it is very common in Japan, less so in Europe, where the greatest incidence is in the Netherlands and France, and is not uncommon in South America. It is probably not so rare in the United States as has been supposed.

Disease in man. The incubation period is six to 12 days, and the high initial fever is followed by nausea and vomiting, epi-

staxis, headache, and muscular pains, and there may be moderately severe bronchitis. Jaundice is not always observed; it occurred in 40 to 60 per cent of the Dutch cases. Convalescence is slow, and weakness may persist for months.

The leptospira are distributed throughout the body in the first week of illness and may be demonstrated in the blood by guinea pig inoculation but rarely in blood smears. After the first week they are present in the urine and may continue to be so excreted for four to five weeks. There is some evidence that the case fatality is reduced by treatment with antiserum. In fatal cases death usually occurs during the second or third week, but occasionally as early as the end of the first week. The case fatality is variable; it has varied from 4.6 to 32 per cent in Japan and has been about 10 per cent in the Dutch cases and as high as 25 per cent in Scotland. At autopsy the leptospira are found in almost all the organs and tissues in those dying during the initial febrile stage, but if death occurs in the second week or later, they are rarely found elsewhere than in the kidneys. Acute renal failure is the commonest immediate cause of death.

Immunity. Recovery from the disease is accompanied by the development of a solid immunity, and a specific lysin is present in the blood which persists in detectable amounts for several years. Agglutinins appear in the convalescent stage, sometimes to very high titer, but the relation of demonstrable antibody and effective immunity is far from clear. Prophylactic inoculation has given encouraging results.

Transmission. The wild rat is the most important animal reservoir of infection, and Schüffner has reported that 40 per cent of tame rats raised for experimental purposes have been found to be infected in Holland. The infection may be detected by darkfield examination of urine and kidney emulsion, and preferably by the inoculation of young guinea pigs with kidney emulsion. Larson has found that a fatal infection may be produced in young mice three to four weeks old.

The proportion of infected rats is variable from one locality to another; in New York about 4 per cent of the rats have been found to be infected, in Japan 40 per cent, in England 30 per cent, in Rotterdam 7 to 40 per cent, and in Philadelphia about 10

per cent. The leptospira are discharged in the urine of infected rats and are transmitted to man via stagnant water contaminated with rat urine. Weil's disease is to some extent an occupational disease and occurs in coal miners, sewer workers, and other working in contact with contaminated water. The majority of the Dutch cases have been in swimmers, bargemen, and fishermen and those who by accident or intent fall into the canals. In one instance a waterborne epidemic occurred in which the water was presumably contaminated by rats from a neighboring sewer. The manner in which the spirochetes enter the body is not known. but very possibly it is though minute cuts and abrasions in the skin or via the alimentary tract.

CANINE LEPTOSPIROSIS

The role of dogs in the transmission of infectious jaundice has been of considerable interest. Dogs suffer from two forms of leptospirosis, the one an acute jaundice ("yellows") similar to acute Weil's disease in man and the other a nonjaundice type known as Stuttgart disease or canine typhus. Lept. icterohemorrhagiae may infect dogs and probably is responsible for the former type of disease, while a canine leptospira, Lept. canicola, produces the latter form. The relative incidence of the two infections in dogs is not known with certainity: some have suggested that as many as 50 per cent of cases of canine leptospiroses are icterohemorrhagiae infections. Lept. canicola infection in dogs appears to be relatively common in the United States, and natural infection with other serotypes occurs also. Surveys² have shown that 13 to 32 per cent of dogs are seropositive, and 11 per cent of the positives are carriers of the infection. In addition to Lept. canicola and Lept. icterohemorrhagiae, which are found most often, dogs in this country are also infected with Lept. grippotyphosa, Lept. pomona, Lept, autumnalis, and Lept, ballum.

SWAMP FEVER

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A leptospiral infection prevalent during the summer and early autumn months in Bavaria, ⁸⁴ Silesia, and the Volga region⁸³ is variously known as swamp fever, Schlammfieber, autumn fever, water fever, field fever, harvest fever, mud fever, and slime fever. It has also been reported from Holland, It is found among field workers following floods or in swampy districts particularly during the hav harvest. Clinically it closely resembles Weil's disease in a mild formthe case fatality is less than 1 per cent - but there is no jaundice except occasionally in the sclera. The causative microorganism. Lept. grippotyphosa (Spirochaeta grippotyphosa), is immunologically distinct from Lept icterohemorrhagiae. The reservoir of infection is the field mouse, Microtus arvalis. The wood mouse, Apodemus sylvaticus, and the bank vole, Evotomys glareolus, are less often infected. A very similar disease is found in the rice fields in Italy, but the etiology appears to be diverse in that a number of species of leptospira have been found. in many instances apparently identical with those found in the Far East.

Reservoirs of infection with many serotypes of leptospira also occur in wild animals, including skunks, raccoons, mongooses, muskrats, opossums, and nutria,³⁶ as shown by both serological evidence and isolation and identification of the leptospira. Human infection may be acquired from these sources, such as Fort Bragg fever, an infection with *Lept. autumnalis*, apparently acquired from raccoons.⁷⁵

Domestic animals, especially swine and cattle, in addition to dogs as described above, also serve as sources of human infection. Swineherd's disease (Bouchet-Gsell disease), found in Switzerland and elsewhere,64,65 is often an infection with Lept. pomona. A number of serotypes have been found in infections of cattle, and in some cases it may be disseminated among the animals through the agency of infected water.43 Human infection from this source tends to be an occupational disease.⁷⁸ In the United States leptospirosis is more widespread than had been supposed, in cattle as well as in the larger wild animals; for example, infected cattle have been found in all counties in Iowa, 109 and a variety of serotypes occur in human infections derived from this and other sources.54

LEPTOSPIRAL INFECTIONS OF THE FAR EAST

A number of leptospiral infections occur in the Far East. They are similar to swamp fever in that they are febrile, jaundice is rare except in the sclera, and the case fatality rate is low. Japanese seven-day fever, or nanukayami, is prevalent in Japan in the autumn. The causative agents is Lept. hebdomadis and is carried by the field mouse, Microtus montebelli. A common sequel to the infection is opacification of the vitreous humor. Hasami fever is a similar disease also prevalent in Japan and caused by Lept. autumnalis (Lept. akiyami A). The reservoir of infection is the mouse Apodemus speciosus, and possibly other species of field mice and rats are infected.

Leptospirosis of heterogeneous etiology is not uncommon in Indonesia. The disease which occurs in Sumatra, known as Rachmat infection, is caused by Lept. autumnalis and is presumably identical with Hasami fever in Japan. Other infections are known by various names, including Andaman A fever, Salinem infection, and the like. The causative organism of Andaman fever appears to be Lept grippotyphosa. The name Lept. pyrogenes has been given the causal agent of Salinem fever. The remainder of the leptospiroses appear to be largely infections with Lept. bataviae, which is found in natural infections in rats, including Rattus norvegicus and R. decumanus (in Japan), and in field mice including Apodemus sylvaticus, Micromys minutus, and M. soricinus.

Mild fevers of leptospiral etiology are endemic in Queensland in Australia, and a number of immunologically distinct varieties of leptospira have been isolated. That designated Lept. pomona is the causative agent of the affection known as Pomona fever, and other species named Lept. australis A and Lept australis B are responsible for febrile disease designated Mossman fever, coastal fever, and the like. Additional varieties have also been reported, but it is not as yet clear that they are distinct species.

Geographical separation of the leptospiroses is not sound, for some of these microorganisms occur in widely separated regions. Thus Lept. grippotyphosa is found in Indonesia and Lept. bataviae and Lept. pomona have been reported as the etiological agents of swamp fever in Italy. Lept. icterohemorrhagiae and Lept. canicola occur in human beings and in dogs, and Lept. pomona has been found in naturally occurring infections of cattle in this country.

SAPROPHYTIC LEPTOSPIRA

Leptospira closely resembling Lept. icterohemorrhagiae have been isolated from water by a number of workers. These nonpathogenic forms have been termed Lept. biflexa in this country and in England, and Spirochaeta pseudoicterogenes by the German workers. Their relation to the pathogenic leptospira is uncertain.

Rat-Bite Fever (Spirillum morsus muris)

There are two distinct kinds of disease which may follow the bite of rats and they are designated rat-bite fever. One, Streptobacillus moniliformis infection, is discussed elsewhere (Chap. Twenty-seven). other, known in Japan as sodoku, is caused by a spiral microorganism discovered by Futaki and his co-workers in 1916. Following the bite of an infected rat, the original wound heals, but, after an incubation period of 10 to 22 days, becomes inflamed and painful. Fever, swelling of the lymph glands, skin eruptions, and other symptoms occur. The fever is of the relapsing type, with paroxysms at fairly regular intervals, usually about once a week, which continue to recur for one to three months or longer. The case fatality varies from 2 to 10 per cent.

The characteristic spiral microorganism

has been found in the swollen local lesions of the skin and the enlarged lymph glands and also in two instances in the circulating blood. Guinea pigs and mice may be infected by the inoculation of blood or fluid expressed from the local lesions. The microorganism is found in about 3 per cent of the house rats in Japan. The rat is not the only vector of the infection, for cases of what is apparently the same disease have been traced to the bite of the cat, dog, pig, ferret, squirrel, and weasel.

The disease has long been known in Japan and has been reported from many localities all over the world. Brown and Nunemaker¹⁷ have found a total of 125 cases reported in the United States from 1916 to the end of 1940. What proportion of the earlier reported cases were Streptobacillus infections

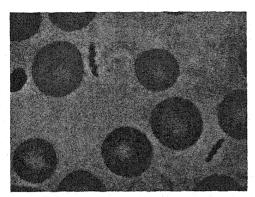


Figure 228. Spirillum morsus muris in blood of an inoculated mouse. (Van Sandt.)

is, of course, problematical. Of 40 cases reported from 1931 to 1940, the spirillum was demonstrated by animal inoculation in 17; of these, 11 resulted from rat bite and the remainder had a history of mouse bite, cat bite or scratch, contact with dogs, or trauma without known animal contact. The natural occurrence of spirochetes in laboratory mice, rats, and guinea pigs is a source of error to be guarded against in laboratory diagnosis by animal inoculation. There is little doubt that the affection is much more common than appears from the records, for the cases occur sporadically and their true nature has often gone unrecognized.67 The infection has been produced in man by artificial inoculation for the treatment of paresis; according to Brown and Nunemaker there are 104 such cases in the English literature.

Not a great deal is known of the immune response to the infection other than that a spirillicidal antibody is produced that is also responsible for immobilization of the spirilla in immune serum. Savoor and Lewthwaite⁹⁵ have observed that a positive Weil-Felix reaction of the OXK type is produced in experimental animals, but the antigen shared with these strains of Proteus is distinct from that which is responsible for the spirillicidal antibody.

The spiral microorganism of rat-bite fever has been variously classified as Spirochaeta morsus muris, Borrelia muris, Spirillum minus, etc. It is shorter (2 to 5 μ in length) than the recognized spirochetes, is relatively rigid, and possesses polar tufts of flagella which give it a rapid darting motion unlike the flexible undulating movements of the spirochetes. The clinical symptoms of the disease are typical of spirochetosis.

Arsenicals have been used chemotherapeutically in the past, and the bacteria are sensitive to penicillin and the broad-spectrum antibiotics; penicillin-tetracycline therapy was found highly effective in one instance. Originally given the name Spirochaeta, this microorganism was found to be identical with *Spirillum minus* found by Carter in the blood of a rat in India. It is, perhaps, best given the independent generic name Spirillum and considered with the spirochetes.

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Chapter Thirty-four

MEDICAL PARASITOLOGY

By ROBERT M. LEWERT, Sc.D.

The division of microbiology termed medical parasitology encompasses the animal agents of human disease. It may seem incongruous to include in "microbiology" organisms such as the larger helminths whose dimensions are measured in meters or liters, as is the case with the cestodes Diphyllobothrium and Echinococcus. There is, however, an entire spectrum of dimensions of parasitic animals, including the intracellular protozoans, only a few microns in diameter. Within the large group of phylogenetically dissimilar animals parasitizing man, parallel evolution has resulted in numerous instances of superficial similarity of form and host-parasite relationship. Physiological interactions with and dependencies on the host occur which we more commonly attribute to bacteria, viruses, and rickettsiae.6, 160

These similarities include such obvious disease production as anemias, dysenteries, encephalitis, and pneumonitis, as well as more obscure effects, such as hypersensitive and allergic responses. However, in contrast to other parasites, phylogenetically related parasitic animals are typified by variety and contrast rather than unifying principle in their disease production, physiology, morphology, life cycles, and other characteristics.

Knowledge of the large parasitic roundworms such as Ascaris and Dracunculus and the large tapeworms and flukes is evident in the earliest writings, and the elucidation of a number of complex parasite life histories and appropriate control measures preceded much of our detailed knowledge of other microbial disease.^{68, 137}

Descriptive parasitology has been a productive division of zoological science, re-

sulting in a tremendous body of knowledge on the complex life cycles and morphology of these forms. This supplied the knowledge of the minute morphologic detail needed for parasite identification for, in contrast to the bacteria, diagnostic, metabolic, culture characteristics were largely unobtainable by usual procedures.21, 98, 140 Investigations of parasite physiology have been difficult because of inability to cultivate most forms free of their hosts. Some demand several hosts, each physiologically necessary to the cyclic development of the parasite, and difficulties of culture might be anticipated. Others of such apparent morphological and cyclic simplicity as the intestinal amebae have also resisted intensive research directed at their cultivation on defined mediums in the absence of other living cells.

For these reasons, techniques of immunization, effective chemotherapy, and practical control measures have lagged behind those developed to combat other disease agents. Consequently, the current world incidence of infection and disease produced by parasitic animals remains high and in some areas is increasing. Even in the case of malaria, which is being attacked on a global scale, annually 1.2 billion individuals live in areas where they are exposed to infection, 200 million cases are reported, and at a conservative estimate 1.5 million deaths result.124, 127 Among the helminths the most important example is the schistosome complex which currently parasitizes approximately 4.5 per cent of the world's population and is increasing its distribution and prevalence in much of its range. 101, 122 The roundworms parasitizing man are so successful that the number of infections

exceeds the world population. Although many of these singly are relatively benign or only slightly debilitating to an otherwise healthy host, multispecies infections are the rule in many areas of the world where their effects are further enhanced by subsistence nutritional levels of the host.

Parasitic animals are of such importance to tropical medicine that this has resulted in the popular misconception that parasitic disease of man is exclusively a problem of the tropics.95, 139 Natural transmission of Ascaris and Trichinella extends into arctic areas, and the classic endemic areas of hydatid (Echinococcus) disease are sheep raising areas, including Iceland.24, 43, 44, 76 Until the relatively recent past the midwestern United States and much of Europe were malarious areas. 62, 127 Although more common in tropical areas, autochthonous amebic dysentery persists in causing individual and occasionally epidemic problems in northern urban communities, 100, 111 while Enterobius and Hymenolepis are dependent on the urbanization of man rather than on climate for survival. In addition, because of increased travel and protracted incubation periods of many parasitic diseases, numerous isolated instances of infection with exotic species are diagnosed in all major northern urban areas.

Disease-producing parasites of man are found among the unicellular animals (protozoa), the worms (platyhelminthes, nemathelminthes, and acanthocephala) and the arthropods. The latter include many species of insects and arachnids of importance as parasites, disease vectors, intermediate hosts, and forms damaging by virtue of their toxic chemical, traumatic, or sensitizing properties. Only those of importance as intermediate hosts of parasites will be included in this chapter. It is not possible here to present material on the arthropods as disease agents and reference is made to various texts on the subject for full coverage. 65, 71 Over 150 protozoan and helminth parasites have been reported from man with approximately one-third of these being common associates. Representatives have been selected for inclusion in this chapter to serve as illustrative examples or for their intrinsic importance in disease production. References for further study and more detailed coverage include standard clinical texts and selected texts and papers cited. 24, 43, 44, 76

The Protozoa

The protozoa are usually thought of as single-celled animals, analogous to the individual cells composing the higher animals. Some authorities, however, prefer to regard them as acellular organisms, not functionally subdivided into cells. Both views have value, for the protozoa evince properties both of single cells and of complete organisms.

Many thousand species of protozoa have been described, though less than 35 well-defined species are known to parasitize man. They constitute an exceedingly heterogeneous group of organisms, varying in size from that of the larger bacteria to several millimeters in diameter, in complexity of structure from a simple, formless cell like Entamoeba histolytica to organisms of far greater intricacy than many metazoa, and in life cycle from the binary fission of Trichomonas vaginalis to the alternation of hosts and of asexual and sexual reproduction exhibited by the malarial parasites. §77, 90, 97

The systematics of the protozoa is complex⁹¹ and no single scheme of classification is uniformly acceptable. Division of the phylum Protozoa into four subphyla plus a small group with uncertain affinities provides a satisfactory simplified method of encompassing the species parasitizing man.

Subphylum Sarcodina (amebae), characterized by the ability to produce from the cell transient finger-like protoplasmic processes (pseudopodia) for the engulfment of food and for locomotion. Many free-living species as well as parasitic forms exist. The parasites characteristically are simple in appearance without morphologically complex organelles and multiply without any known sexual stages. All have a motile trophozoite stage during which multiplication is by binary fission. Many species also form a cyst in which stage two or more nuclear divisions may take place preceding the multiplication effected when the cyst infects the host. Amebae of man are primarily found in the digestive tract.

Subphylum Ciliophora (ciliates), having numerous, short, bristle-like, cytoplasmic locomotor organelles termed cilia. Most have two types of nuclei (macronucleus and micronucleus) with multiplication by binary fission and with conjugation in some forms.

There are many free-living, commensal and symbiotic species with but a single form, *Balantidium coli*, occurring as a bona fide parasite of man.

Subphylum Mastigophora (the flagellates), characteristically having throughout or at some point in their life cycle relatively long, fllamentous protoplasmic processes used in locomotion. These flagella may be multiple or single. Those intracellular parasitic stages lacking flagella may be recognized by a parabasal body, a rod-like structure associated with the flagellar origin. Division is by binary fission. Evidence for a sexual cycle in the hemoflagellates has been presented but is not wholly acceptable. Numerous free-living, parasitic, and commensal species exist. Flagellates parasitizing man are found in the intestinal and genital tracts, free in the circulation, and as intracellular parasites primarily of the lymphoid-macrophage system.

Subphylum Sporozoa, an artificial polyphyletic grouping of parasites characterized by the lack of well-defined organelles of locomotion and with an alternation of sexual and asexual reproduction. All members of this group are parasitic and many produce structures of varied morphology which have been designated as spores and which contain one to many infective individuals termed sporozoites. The malaria parasites (Plasmodia) and the intestinal coccidia of man belong

to this group.

A separate group superficially similar to the sporozoans, of uncertain affinities, has been characterized as a class, Toxoplasmasida, having no spores and with asexual reproduction. This group would include Toxoplasma and Sarcocystis of man as well as a variety of parasites of other vertebrates.^{90, 91}

To supplement this skeleton classification and the material to follow, a number of basic texts and taxonomic studies are available.^{87, 97}

THE INTESTINAL AMEBAE (Sarcodina)

Entamoeba histolytica. Lewis in 1870 and Cunningham in 1871 first reported amebae, probably the nonpathogenic Entamoeba coli, in human feces. Lösch in 1875 described what were apparently E. histolytica in the stools and intestinal ulcers in a fatal case of dysentery. He found similar ulcers containing the amebae in an artificially infected dog.

Characteristics and life cycle. The active ameba as seen in the intestinal ulcers of dysentery cases is a granular colorless or pale greenish mass of cytoplasm, 15 to 50 (usually 20 to 30) μ in diameter. It has no definite shape. Locomotion is accomplished by the sudden extrusion of clear projections of cytoplasm, the pseudopodia, the remainder of the cell body following these pseudopodia in a flowing motion. The granular cytoplasm often contains red blood

cells or debris of tissue cells in various stages of digestion. These are the food engulfed by the ameba. The nucleus may be visible as a delicate ring of granules. Reproduction in this stage is by binary fission, the nucleus undergoing a type of mitosis and the cytoplasm then dividing to produce two daughter amebae like the original. In organisms fixed and stained with hematoxylin the nuclear structure is characteristic, consisting of a thin peripheral layer of fine black granules and a central small black dot, the *karyosome* (Fig. 229). The entire nucleus is generally 4 to 5 μ in diameter.

Infected persons with diarrhea or dysentery pass active ameboid parasites in their stools. In the intestinal lumen of a carrier, however, the ameba loses its ingested food particles, shrinks to a diameter of 10 to 20 μ (rarely less), rounds up and becomes essentially nonmotile, sending out only an occasional pseudopodium. This is the precystic stage, which soon secretes about itself a clear wall, becoming the partially resistant cyst. In its passage down the intestine the cyst continues to develop, acquiring a vacuole of glycogen and one or more ovoid rods of black-staining material, the chromatoid bodies. The nucleus divides into two and then four, all resembling the nucleus of the active ameboid stage though considerably smaller. In the mature cyst the glycogen vacuole soon disappears, and the chromatoid bodies persist at most for a few days.

The cyst is the infective stage, since most or all of the active ameboid stages are destroyed by gastric juice. As studied in culture, excystment consists of the emergence of a four-nucleate organism which by a complicated division process produces eight small amebae. These are the stages which initiate a new infection.

Boeck and Drbohlav in 1925 cultivated E. histolytica in Locke's solution and inactivated serum tubed over egg slants. Various modifications have been introduced, the most important being the addition of powdered rice starch, which the amebae ingest avidly. Cleveland and Collier used liver infusion agar slants overlaid with serum-saline. Balamuth devised a valuable monophasic medium consisting of buffered aqueous egg yolk infusion, with or without liver extract.

These mediums do not permit pure culture, for the amebae are dependent on living

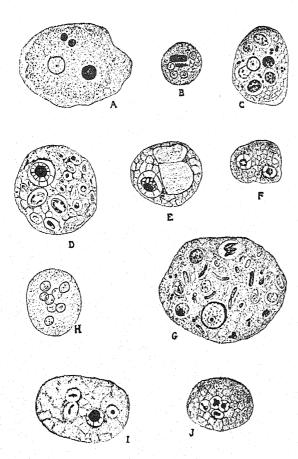


Figure 229. The amebae living in man. A. active ameboid form of Entamoeba histolytica containing three red blood cells. B, mature quadrinucleate cyst of same containing two chromatoids. E. hartmanni is similar but has smaller nuclei. C, active ameboid form of Entamoeba gingivalis. D, active ameboid form of Iodamoeba williamsi containing many intestinal bacteria. E, cyst of same showing large double vacuole which in life was filled with glycogen. F, ameboid form of Dientamoeba fragilis containing two nuclei, G, large active ameboid form of Entamoeba coli containing intestinal bacteria and debris, H, mature octonucleate cyst of same. I, ameboid form of Endolimax nana. J, mature quadrinucleate cyst of same. $A, B, C, G, and H, about \times 1300 (Dobell), D, E, F,$ and $J_1 \times 3000$ (Taliaferro and Becker). $I_1 \times 3000$. (Taliaferro in Hegner and Taliaferro's Human Protozoology, courtesy of The Macmillan Company.)

bacteria for growth. The protozoan Trypanosoma cruzi has also been utilized as an associate for culture of the amebae. Shaffer and Frye obtained amebic growth in the presence of antibiotic-inhibited bacteria. Cultivation of one strain of E. histolytica in a cell-free system is now possible and is being utilized for antigen preparation and immunological studies. This is the first extensive truly axenic cultivation of this species, although not vet applicable to all strains.35 The related tissue-invading, pathogenic parasite of snakes, E. invadens, has been cultivated successfully in a cell-free liver extract medium. E. histolytica is a strict anaerobe and requires a pH between 6.5 and 7.0 approximately.99

Amebiasis and amebic dysentery. The amebae normally inhabit the large intestine. While some workers believe that they proliferate only in the tissues, the evidence indicates that they may also multiply in the intestinal lumen, living, as in culture, on a diet of microorganisms. ⁸⁶

In the wall of the colon the parasites are found in necrotic, usually noninflammatory

lesions varying from minute erosive patches to more or less extensive, undermined ulcers of the submucosa. The abundance and severity of the lesions determine the clinical picture. The great majority of infected individuals are carriers exhibiting no symptoms. Clinical cases range from moderate diarrhea to acute dysentery with passage of blood and mucus, extreme weakness, and, not infrequently, death. A varying proportion of individuals with amebic dysentery show necrotic abscesses of the liver, or may show a generalized hepatitis of amebic etiology. Initially the liver abscesses are bacteria-free but may become contaminated secondarily.89,94 These also occur occasionally in individuals who give no history of intestinal symptoms of infection. Lung abscesses, usually produced by extension from the liver through the diaphragm, occur in a small number of individuals, and abscesses have been reported in practically every organ of the body.

The entire question of host-parasite relationships in amebiasis including disease production is complex and controversial.42,66,102 The lack of agreement even in the morphological bases for diagnosis of the species causing amebic disease has also complicated the picture.15, 20, 121 Strains of the species vary in their morphology and in their pathogenicity for man. 67, 108, 109 Some rapidly lose pathogenicity in culture, and human pathogens may or may not be pathogenic for laboratory animals.64 Strains of human origin, pathogenic for animals, have been shown to be completely innocuous in human voluteers.9 Some investigators have presented evidence that the pathogenicity of amebae is directly related to the pathogenicity of the bacterial associates, while others refute this. The dependence of most strains of amebae in culture on bacterial associates and the inability of amebae to produce extensive lesions in bacteria-free guinea pigs lend weight to the concept of the need for synergistic bacterial associates in amebiasis.93, 115 However, as mentioned earlier, sterile amebic abscesses are found. The suggestions that individual variation in resistance and that nutritional factors are of primary importance in the severity of the disease have supporters but are also disputed.37, 49 Attempts have also been made to associate invasiveness of strains to the presence or lack of protease or mucopolysaccharidase activity with equivocal results.80, 109

IMMUNITY. There is no direct evidence. of acquired immunity to infection with E. histolytica. Natives of hyperendemic regions show acute dysentery less frequently than aliens, possibly as a result of acquired immunity. Serum antibody response to the infection is evidenced by the complementfixation test, which is positive in over 80 per cent of infected individuals. Sera from infected individuals will inhibit to varying degrees the ability of amebae in culture to ingest erythrocytes or can be shown to immobilize their locomotor activities. 138 Antibodies from infected individuals when labeled by fluorescein have considerable specificity for the infecting species and a degree of strain specificity.58,59 Immune precipitates in a gel diffusion system on a micro scale also show species specificity. A diagnostic test using an indirect hemagglutination technique has also been devised and is being utilized in some laboratories. 86, 105 As with similar systems for parasitic infections it is not possible at present to correlate these diagnostic serologic phenomena with functional immunity.

DIAGNOSIS. Diagnosis of infection with E. histolytica depends upon the finding of characteristic organisms in the stools and their morphological differentiation from the nonpathogenic amebae occurring in human feces. Cultivation has been utilized, but in cultures E. histolytica resembles the nonpathogenic E. coli so closely that differential diagnosis is often very difficult. Direct smears of fresh loose stools in warm saline may reveal the active ameboid stages. They may also be found in material taken directly from lesions in the lower bowel with the aid of the proctoscope. The M.I.F. stain of Sapero, Lawless, and Strome is valuable for temporary preparations, although permanent smears stained with iron hematoxylin give the best differentiation. In well-formed stools cysts may be expected, and these are most easily identified in iodine solutions, such as D'Antoni's iodine. The formalin-ether concentration method of Ritchie (for cysts) and the M.I.F. concentration method of Blagg et al. (for both cysts and trophozoites) are excellent for diagnosis of the light infections which may be missed by direct examination. The P.V.A. preservation technique of Brooke et al. permits shipment of material to a central laboratory for examination. 15 E. histolytica appears irregularly in the stools and, whatever the technique, repeated examinations are often necessary to establish a diagnosis. It should be re-emphasized that recovery of the organisms is only the first step in diagnosis of E. histolytica infection. The final step is the identification of the parasites. a task requiring abundant experience. Some concept of the variation between individuals can be gained by examining photomicrographs.^{21, 140} However, considerable experience is needed to exclude the commensals and smaller related forms such as E. hartmanni. The varied symptomatology and severe nature and importance of amebic disease necessitates the development of a more objective diagnostic method than presently exists. Axenic cultivation procedures and immuno-cytochemical methods being studied may produce this in the future.

CHEMOTHERAPY. Emetine has a favorable effect on the symptoms but rarely eliminates the infection from the intestine. Several iodine-containing drugs, notably chiniofon and Diodoquin, and arsenicals, especially carbarsone and Milibis, are effective in eradicating the amebae.

Several antibiotics are useful in treat-

ment. Chlortetracycline is moderately efficient, and oxytetracycline cures about 90 per cent of cases. Chloroquine and Camoquin, while useless in intestinal amebiasis, are highly curative in extraintestinal infection

Epidemiology and control. The active ameboid stages of E. histolytica die quickly after exit from the body, for they are very susceptible to drying and to changes in temperature and salt concentration. Since they are destroyed by gastric juice, they are not usually infective when swallowed. The amebic dysentery patient is therefore practically harmless as a source of infection, since only the ameboid stages occur in his stools. The cysts passed by carriers, while not at all comparable to bacterial spores in resistance, show considerably less susceptibility to conditions outside the body than do the ameboid stages. Studies utilizing cysts from culture tested for viability by cultivation show survival of several months in water at 0° C., three days at 30° C., 30 minutes at 45° C. and five minutes at 50° C. Cysts of E. histolytica are somewhat more resistant to chlorine than are enteric bacteria. It is believed that ordinary residual chlorine concentrations are unable to destroy amebic cysts, but that hyperchlorination is effective. In this connection it should be borne in mind that in the Chicago amebic dysentery outbreak of 1933 there was abundant circumstantial evidence that the infection was spread by water containing sufficient residual chlorine to kill intestinal bacteria.100

In general, the spread of E. histolytica resembles that of intestinal bacterial infections, utilizing any means by which fecal contamination reaches the human mouth. Most important are drinking water, food handlers, and houseflies. Swimming pools, though not definitely incriminated, are a potential source of infection. Viable cysts have been found in the droppings of houseflies one to two days after exposure, and flies have been held responsible for at least one important outbreak. The 1933 epidemic in Chicago referred to above was traced to local sewage contamination of drinking water in two hotels. This epidemic, in which 1409 cases were discovered and there were 98 deaths, was the first major outbreak in a city of the temperate zone. It directed medical attention to a problem which had been considered important only in the

tropics. As recently as 1955 a similar outbreak occurred in South Bend, Indiana, with contaminated drinking water as the source, re-emphasizing the fallacy in considering amebiasis an exotic infection.¹¹¹

The distribution of E. histolytica is worldwide, but temperate regions have usually a low incidence of infection. Surveys indicate a general infection rate in the United States of 4 to 10 per cent, though in some southern localities the incidence has approached 40 per cent. These figures include E. hartmanni (see below) and are probably about twice the incidence of E. histolytica alone. In the tropics the carrier rate is generally very high, often exceeding 50 per cent. Human carriers are the only important source of infection, although E. histolytica occurs naturally in lower animals, particularly rats, dogs, and monkeys. The most widely used experimental animals are dogs, cats, monkeys, rats, guinea pigs, rabbits, and hamsters.67

Control of the spread of *E. histolytica* is not basically different from that of other human enteric infections. The high incidence of carriers not known to have had clinical dysentery complicates the problem, but it is ultimately a matter of prevention of access of human feces to the mouths of susceptibles.

Other species of amebae parasitic in man. Six other species of amebae live in the human intestine. With the exception of *Dientamoeba fragilis* these are nonpathogenic and are of medical interest only because they must be differentiated from *E. histolytica*.

E. hartmanni, long considered a small race of E. histolytica, has recently been shown to be a distinct species. It differs from E. histolytica in size, the cysts being always less than 10 μ in diameter, and in size of the nuclei, which are slightly more than half the diameter of those in E. histolytica. Usually it is not distinguished from E. histolytica in diagnostic work, but in a few surveys it has been found to make up about half the infections of the histolytica type. It is nonpathogenic and can be cultured only with difficulty.

Entamoeba coli, a common species, differs from E. histolytica in several characters. The stained nucleus shows thicker peripheral chromatin blocks and a larger and usually noncentral karyosome. The ameboid stage is sluggishly and usually nonprogres-

CILIOPHORA 775

sively motile with blunt, slowly extruded pseudopodia. It ingests bacteria and other particles but rarely red blood cells. The spherical cysts average somewhat larger, 10 to 33 μ in diameter, contain eight nuclei in diameter, contain eight nuclei in the mature stage and may show chromatoid bodies with pointed or "splintered" ends.

E. polecki, a parasite of hogs and monkeys similar to E. coli but producing uninucleate cysts, has been found very rarely in man.

Endolimax nana is smaller, 6 to 15 μ in diameter in the ameboid stage. The stained nucleus shows no peripheral chromatin but a very large, nearly central karyosome. Movement is sluggishly progressive, and bacteria are ingested. The spherical or ovoid cyst is 5 to 14 μ in diameter, containing one to four minute nuclei and sometimes small spherical or rod-like chromatoid bodies.

Iodamoeba biitschlii measures 8 to 20 μ in diameter in the ameboid stage. The stained nucleus shows a large central karyosome surrounded by a layer of granules. Movement and inclusions are like those of $E.\ coli.$ The cyst is irregular in shape, 5 to 20 μ in diameter, and contains one or rarely two nuclei. Minute granules may be seen, but the most striking feature of the cyst is a large glycogen mass, staining dark brown with iodine.

Dientamoeba fragilis, seldom reported in the general population though sometimes common in institutions, is a very small form, 5 to 12 μ in diameter. It usually shows two nuclei, each containing a large multiple karyosome. It moves actively, ingesting bacteria. It has been associated with acute dysentery, and it has been suggested that it may produce low grade constant irritation with fibrotic changes producing an appendicitis.144 It is considered by some protozoologists to be an aberrant flagellate rather than a true ameba. No cyst stage is known and the binucleate trophozoite, as its name suggests, is readily destroyed. One investigation suggests that D. fragilis is transmitted via the egg of the pinworm of man, Enterobius vermicularis.²² Precedent for this is found in the cycle of Histomonas meleagridis, an ameba-like, pathogenic flagellate of the turkey which is transmitted through an Ascarid

E. gingivalis, probably the first parasitic ameba seen, was reported by Gros in 1849 from the tartar between the teeth. It has no

known cyst stage and is apparently transmitted in the ameboid stage by contact. Formerly suspected of an etiological role in pyorrhea, it is now considered harmless.

CILIOPHORA

Balantidium coli is the largest protozoan parasite found in the intestine of man, reaching a size of $150 \times 120 \mu$. The organism may cause an acute bloody dysentery similar to amebiasis, commonly penetrating into the muscularis mucosa and occasionally perforating the intestine.4 Carrier infections without symptoms occur in man, and the world incidence is estimated at less than 0.7 per cent. Swine are almost universally infected and human infection usually derives from food or water contaminated with the resistant cysts from this source. Moderate to severe pathology is produced in other primates, and infections naturally occur in the rhesus monkey, chimpanzee, dog, and Norway rat. Balantidium may be cultivated on a variety of mediums. Carbarsone is an effective chemotherapeutic agent, although Diodoquin and the tetracyclines have also been used successfully in treatment.

INTESTINAL FLAGELLATES (Mastigophora)

The mastigophora of the digestive tract and genital organs, illustrated in the accompanying figure, are typically lumen parasites. The common cosmopolitan species of enteric flagellates, Chilomastix mesnili, Trichomonas hominis, and Giardia lamblia, are host-specific although a number of less common and less specific coprophagic forms are found in man. None are generally considered as important pathogens. However, Giardia is commonly associated with erosion of the epithelium of the duodenum and also with an irritation of the gallbladder. Intense infections in children are considered by some to cause dysentery, celiac syndrome, or a sprue-like condition.32 The parasites and the associated intestinal symptoms disappear following treatment with atabrine.

Trichomonas vaginalis is a cosmopolitan common parasite of the vagina and male genital tract. Infection is commonly symptomless but may produce in the female a

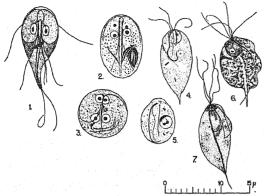


Figure 230. Flagellates of the human intestine and genital tracts, hematoxylin stain. I, Giardia lamblia trophozoite. 2 and 3, G. lamblia cysts. 4, Chilomastix mesnili, trophozoite. 5, C. mesnili, cyst. 6, Trichomonas hominis. 7, T. vaginalis. (Mackie, Hunter, and Worth: A Manual of Tropical Medicine. 2nd ed.)

severe vaginitis and in the male may occasionally be associated with an urethritis. Transmission occurs primarily through sexual intercourse, as the parasite has no resistant stage. Variation in virulence is related to strain of the parasite. It is interesting that infected individuals also are usually infected with the nonpathogenic buccal flagellate, *Trichomonas tenax*. 19

Flagellates related to the above such as *Trichomonas foetus* may be highly pathogenic. The latter, also transmitted by coitus, causes contagious abortion in cattle. *Trichomonas gallinae*, a common parasite of the domestic pigeon, has virulent pathogenic strains producing lesions in the upper digestive tract and associated structures. Virulence transformation of avirulent strain forms treated with homogenates of pathogenic strains has been reported for this species.⁷⁰

Chilomastix and Giardia are spread by ingestion of material contaminated with human feces containing their cysts. Trichomonas has no resistant cyst but the trophozoite may remain viable for more than an hour on dry fomites.

HEMOFLAGELLATES (Mastigophora)

The flagellate protozoa occurring in the blood stream or as intracellular parasites of

man are termed hemoflagellates. All are members of the family Trypanosomidae with seven species found in man, viz., Trypanosoma rhodesiense, T. gambiense, T. cruzi, T. rangeli, Leishmania donovani, L. tropica, and L. braziliense. All have probably evolved from parasites of the intestinal tract of insects and those parasitizing man still require blood-sucking insect vectors or intermediate hosts for normal transmission.⁵¹ During their life cycles hemoflagellates display a variety of morphological types in regular stages. Since these types have been associated with particular genera the stages are named for the genera they resemble. As indicated in the accompanying figure these are the trypanosomal, crithidial, leptomonad, and leishmanial forms. The trypanosomal form is characterized by having a flagellum arising from the posterior of the cell, attached to the cell by an undulating membrane running the length of the body, and ending anteriorly as a free flagellum. In the crithidial form the flagellum arises just anterior to the nucleus with the undulating membrane being correspondingly reduced. The flagellum in the leptomonad stage originates near the anterior end of the body with no associated membrane, while in the ovoid leishmanial stage no free flagellum is present. The structures associated with the point of origin of the flagellum, the kinetoplast and basal granule, may still be seen in the leishmania and are of diagnostic importance.

The medically important hemoflagellates fall into three groups in two genera. Trypanosoma gambiense and related forms exhibit the trypaniform stage in the vertebrate host and trypaniform and crithidial stages in the invertebrate. T. cruzi exhibits all four stages in the vertebrate host, trypaniform and crithidial stages in the invertebrate. Members of the genus Leishmania exhibit only the leishmaniform stage in the vertebrate and the leptomonad stage in the invertebrate host.

Reproduction of all forms is by binary fission. The nucleus, blepharoplast, and parabasal body divide, a new flagellum arises from one blepharoplast, and the cytoplasm divides longitudinally to produce two daughter cells. In the species with which we are concerned, the leishmaniform stages are intracellular in the vertebrate host. The flagellated stages inhabit body fluids of vertebrates or the alimentary tract of insects.

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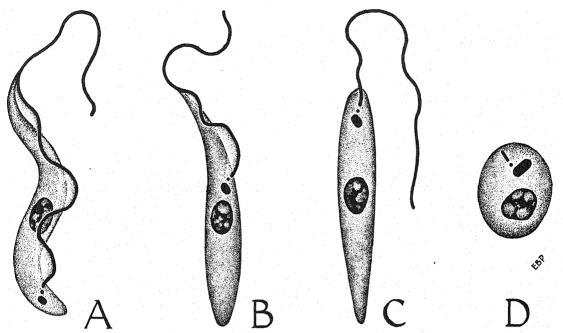


Figure 231. Morphological types of hemoflagellates. A, trypanosomal stage. B, crithidial stage. C, Leptomonas stage. D, Leishmania stage.

THE TRYPANOSOMES

Trypanosoma gambiense. Ford observed, in the blood of a Gambian native, a flagellate which was described by Dutton in 1902 as T. gambiense. In 1903, Castellani observed similar flagellates in the cerebrospinal fluid of a sleeping sickness patient in Uganda. Bruce and Nabarro, in 1903, transmitted T. gambiense to monkeys with a tsetse fly, and Kleine, in 1909, showed that the parasites underwent a cyclic development in the fly.

Characteristics and life cycle. In the blood and lymph of early cases of African sleeping sickness T. gambiense exhibits the trypaniform stage, which in fresh preparations may be seen wriggling among the erythrocytes. It measures 15 to 40 μ in length, varying from short broad forms with no free flagellum beyond the undulating membrane to long thin forms with a free flagellum. Reproduction, as in all members of the family, is by binary fission. The transmitting insect, a tsetse fly (Glossina), becomes infected by ingestion of the parasites in infected blood. In the stomach of the fly those flagellates which survive multiply as trypaniform and crithidial stages, first in the crop, stomach, and intestine and later in the salivary glands.

Here they form infective parasites, which are injected by the bite of the fly 20 days or more after its infecting blood meal.

On NNN medium, a concentrated blood agar, the parasites multiply to a limited extent. Continuous cultivation has been

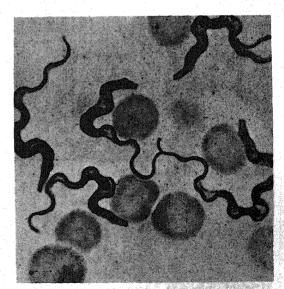


Figure 232. Trypanosoma gambiense in stained blood film. About × 2000.

achieved in some instances, but it is too difficult to be of practical value. Abundant multiplication occurs on the chorioallantoic membrane of the developing chick embryo. Development of a related species, *T. vivax*, to the infective stage in tsetse fly tissue culture has been accomplished.¹⁵¹

African sleeping sickness. The incubation period following an infective tsetse fly bite varies from two or three weeks to several months. The first stage of the human disease is a systemic infection in which the parasites are found chiefly in the blood but also in the lymph. Irregular fever is generally the first symptom. Somewhat later, as the parasites come to predominate in the lymph nodes, these organs and the spleen are enlarged, and anemia and wasting are seen. Cardiac injury may be prominent, and edema is often present. This stage gradually gives way to the sleeping sickness stage, in which the parasites are most abundant in the cerebrospinal fluid, occurring less commonly in the lymph. The central nervous system exhibits the prominent lesions, typically resulting in somnolence, apathy, and weakness, less commonly in mania and other violent manifestations. Coma and death are the final outcome. Typically the course is of several months' duration, but in parts of East Africa the disease develops more rapidly, invasion of the central nervous system occurs early, and death frequently results from cardiac injury before the typical sleeping sickness stage is reached. In the blood of laboratory animals infected from cases in this area, about 5 per cent of the flagellates are short, showing posterior nuclei; this is true only rarely in the parasites from other areas. Because of these differences in morphology and pathogenicity and the fact that transmission involves different species of tsetse flies, Stephens and Fantham separated the East African parasites as a different species, T. rhodesiense. Most authors now consider this a local race of T. gambiense.33

IMMUNITY. Spontaneous recovery is said to occur occasionally in African sleeping sickness. Except for this there are no data available on natural or acquired immunity in man. In rats and mice T. gambiense and related parasites produce rapidly fatal parasitemia, while in guinea pigs and some other hosts they show a characteristic relapsing type of infection. After a period of unchecked multiplication, the organisms are rapidly destroyed, only to reappear in a few

days and repeat the cycle. An antibody arising in the serum of a guinea pig at the time of crisis destroys parasites of the strain present before the crisis, while the strain present after flagellates have reappeared is resistant to this antibody. This phenomenon has been observed in animals infected originally with a single parasite, indicating that the change in antigenic structure cannot depend on selection alone but must involve adaptive modification of the parasite.

DIAGNOSIS. Various serum reactions are observable in African trypanosomiasis but are not dependable for diagnosis, chiefly because of the antigenic lability of the parasites. Laboratory diagnosis depends on the finding and morphological identification of the organisms in blood, lymph node juice, or cerebrospinal fluid. Dry smears stained with blood stains, such as Wright's, are used, either of the whole fluid as obtained or of centrifuged specimens. In the case of blood, thick or thin films may be used. The parasites are usually scarce, and a negative finding in any one of the body fluids is of little significance.

Epidemiology and control. Rare instances of probable venereal infection have been reported. However, the normal transfer of infection occurs by the bite of tsetse flies as described above. Two species of flies are of most importance, Glossina palpalis in West Africa and G. morsitans in East Africa. where the more virulent Rhodesian form of the disease occurs. Other species of importance are G. tachinoides, G. swynnertoni, G. pallidipes, and G. austeni. These insects are relatives of the common housefly. They are limited to tropical Africa and a small area in South Arabia, and the human disease is confined to their range. They resemble the housefly in appearance except for the long narrow proboscis, which is held straight forward from the head, and the manner of folding the wings at rest, flat on the back with one directly above the other. Both males and females bite and can transmit the disease. They bite exclusively by day. The larvae develop completely in the body of the female, are deposited singly on loose soil or sand in well-shaded places, and quickly burrow into the soil to pupate. After four to eight weeks the adults emerge. G. palpalis breeds almost entirely near water and is thus more limited in local distribution than G. morsitans, which is less dependent on shade and moisture.

The significance of wild animals in the spread of African sleeping sickness has long been disputed. Various large wild animals, especially the sitatunga antelope, harbor the flagellates, usually without symptoms, and they have been shown to maintain the infection for long periods in the absence of human reservoirs. However, most authorities agree that man is usually the source of infection. The incidence of human infection varies widely. It is not commonly higher than 2 per cent at present, though in the past villages were observed with infection rates as high as 50 per cent, and catastrophic epidemics have occurred.

The variety of control methods in use testifies to their relative ineffectiveness. Several valuable drugs are available for treatment of the human infection, a method whose success is proportional to the local significance of man as a reservoir of infection. Tryparsamide and other arsenicals are effective in all stages of the disease, though less so in the Rhodesian form, where Bayer 205 (suramin) is more active. This drug is useless in the sleeping sickness stage of the disease but is effective during the first stage and has the particularly valuable property of protecting against infection for at least three months after administration. A new series of diamidine compounds represented by pentamidine shows genuine promise. Pentamidine cures existing cases and protects against infection for at least six months after administration. Hundreds of thousands of African natives have received this drug in mass treatment campaigns, and the resulting reduction in incidence of infection suggests that this is the most effective control measure available.

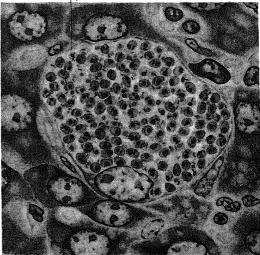
In some areas mass destruction of the reservoir game animals has been attempted, but the success of this measure is doubtful. Where the human and animal infection rates are particularly high, wholesale removal of human populations has been carried out. Control of migrants, a perpetual problem in much of Africa, is utilized in some districts in an effort to minimize spread of the disease.153

The life cycle and complex behavior of the tsetse flies make their control exceedingly difficult. Traps and hand-catching have greatly reduced the fly population in some regions. Inspection and fumigation of vehicles have been used to limit the spread of tsetse. The best single method is clearing of forest and brush, particularly along streams and around villages, which destroys the breeding and resting places of the flies.

Trypanosoma cruzi. Chagas, in 1909, discovered intermediate stage flagellates in the hindgut of the bug Triatoma megista in Brazil. He showed the infectivity of these flagellates for mammals and later found trypaniform stages in children with a characteristic disease, now known as Chagas' disease. He described the organism as Schizotrypanum cruzi, but it is now called T. cruzi by most authors.

Characteristics and life cycle. The parasites as seen in the blood of early cases of Chagas' disease are of the trypaniform type, fundamentally similar to those of T. gambiense but smaller, about 20 μ in length, and with the posterior end pointed. No multiplication occurs in the blood. The predominant phase in man and experimental animals is a small, rounded nonflagellate stage, 3 to 5 μ in diameter, occurring in clumps in various tissue cells-heart muscle, macrophages, and endothelium. These organisms have a nucleus and parabasal body as described above. They multiply by binary fission, producing dense clumps of organisms which fill the infected cells. Small numbers of trypaniform stages are apparently continually produced and shed into the circulating blood from the nests of intracellular nonflagellate parasites.

Infected Kupffer cell



Liver cell

Crypt epithelium ---

Figure 233. Leishmaniform stage of Trypanosoma cruzi Tulahuén strain in Kupffer cell of liver. (Taliaferro The second secon

Various bugs of the family Reduviidae acquire the infection from a blood meal containing the trypaniform stages. These become crithidial flagellates in the midgut and hindgut, where they multiply to produce infective trypaniform flagellates two to three weeks after the infecting blood meal. These organisms are shed in the feces of the bug. They infect man by entering the wound caused by the bite of the infected bug or by penetrating the mucosae, particularly of the mouth or eye.

Continuous culture of *T. cruzi* on NNN medium and in simpler broth mediums is relatively easy. The parasites multiply as trypaniform and, predominantly, crithidial flagellates. The nonflagellate stages have been grown in tissue cultures of macro-

phages from infected animals.

Chagas' disease. Chagas described the infection from a region having serious endemic goiter, and most of his cases had marked thyroid pathology. It has since been shown in other regions that the goitrous manifestations he described are not part of Chagas' disease. The incubation period is one to two weeks. Irregular fever and edema, particularly of the eyelids (Romaña's sign), characterize the acute phase, and there is considerable enlargement of lymph nodes, spleen, and liver toward the end of this period. The acute disease is rare except in small children, in whom occur almost the only deaths attributable to the infection. The chronic disease, which occurs in adults or following the acute stage in children, varies in symptomatology with the localization of the parasites but is most often characterized by myocarditis. 123

In addition to the myotropic strain, a reticulotropic strain (Tulahuén) occurs in Chile. Enlargement of the spleen and liver as well as engorgement of bone marrow may give a pathology resembling visceral leishmaniasis. Transplacental infection may occur but rarely, and the conditions of megacolon and megesophagus found in Argentina and Chile are attributed to parasitism by this species. 46, 136

IMMUNITY. Evidence concerning acquired immunity is lacking in the human disease, but experimental animals are immune to reinfection after recovery. Serum from such animals partially protects against experimental infection. The serum of human cases fixes complement in the presence of extracts of infected tissues or cultures of

T. cruzi, and the complement-fixation test (the Machado reaction), or any of several modifications of it, is widely used for laboratory diagnosis. A relatively nonpathogenic strain⁵⁷ of T. cruzi has been found to occur as a parasite of wild mammals in Maryland.¹⁶¹ Infection of mice with this strain confers immunity to superinfection with the more virulent strains.⁸⁴

DIAGNOSIS. Laboratory methods for diagnosis other than the complement-fixation reaction depend on the demonstration of the parasites. During the early stages of the human disease trypaniform flagellates may be found in stained blood smears. Later, indirect evidence of the presence of parasites may be obtained by blood culture or by "xenodiagnosis" (host diagnosis), in which laboratory-reared bugs become infected after feeding on a case. Fluorescent antibody methods, although not in widespread use, have also been shown to be of practical diagnostic value.

Epidemiology and control. Infection is normally acquired from infected bugs as described above, although occasionally the disease is acquired by direct contamination of mucosae, as in the congenital infection of infants. The chief vectors are Triatoma (Mestor) megista and Rhodnius prolixus, but about 40 species of the family Reduviidae have been incriminated. These insects are members of the order Hemiptera, or True Bugs, to which belong also bedbugs and many others. They are large insects with an elongated, cone-shaped head. Most species are predatory on other insects, but some live on vertebrate blood. They inhabit the nests of various animals and may occur in human houses of poor construction, where both the male and female insects commonly bite sleeping persons about the mouth or eyes. The total life cycle, involving egg, larval, nymphal and adult stages, occupies six to 10 months.

The human disease is widespread in South and Central America but of low incidence in most areas. It has been reported from every country in the Western Hemisphere, with the exception of Canada, Honduras, and the Guianas, as a sylvatic infection. *Trypanosoma cruzi* has been reported in Arizona, California, Georgia, Louisiana, Maryland, New Mexico, and Texas. 165 In addition two indigenous cases have been recorded in humans in Texas. 166 The possibility that more extensive infections occur

than are reported is suggested by the fact that patients in the eastern United States suffering from a diffuse myocarditis have been found to react positively to serological tests for *Trypanosoma cruzi*.⁴¹

Successful control of Chagas' disease has not been attained. The most promising methods involve destruction of infected domestic animals and improvement of human houses to exclude the insects and the reservoir hosts. There is no successful chemotherapy for Chagas' disease, the drugs useful in African trypanosomiasis being inactive in this infection.

Trypanosoma rangeli. Tejera, in 1920, described from a vector of T. cruzi in Venezuela a flagellate which he named T. rangeli. In 1942 this was obtained from humans by xenodiagnosis and since that time has been reported widely from Central and South America in the areas in which Rhodnius prolixus, its vector, occurs. Trypanosome division takes place in the peripheral circulation of man, dog, and monkeys with no leishmaniform stages demonstrated. No clinical manifestations are known and two experimental infections in man were without symptoms. In contrast to T. cruzi, T. rangeli is transmitted to man through the bite of the insect.

Other species of Trypanosoma. A number of important diseases of domestic animals are caused by species of Trypanosoma similar to T. gambiense. Nagana is a rapidly fatal disease of the horse family, and to a less extent of cattle and dogs, occurring in a wide area of East Africa. It is caused by T. brucei, an organism very similar to the Rhodesian form of T. gambiense. A number of other species transmitted by tsetse flies are important disease agents of animals in Africa. T. evansi causes a disease of horses, camels, and mules known as surra, which is widespread in Asia, extending to Russia, Arabia, and Madagascar.

T. lewisi of rats elicits an interesting antibody, which inhibits reproduction of the parasites without killing them. This antibody, ablastin, interferes with nuclear division, and the production of nucleoprotein by the parasite. 147

THE LEISHMANIAS

Leishmania donovani. Leishman and Donovan, in 1903, described oval parasites of the macrophages in cases of kala-azar in

India. These were recognized as mastigophora when Rogers showed they developed into motile flagellates in culture.

Characteristics and life cycle. In the human disease, kala-azar, the parasites occur as nonflagellate stages, 3 to 5 μ long, in macrophages, where they resemble the nonflagellate stages of T. cruzi as shown in the accompanying figure. They multiply by binary fission until the cytoplasm of the host cell is crowded, when they escape to infect new cells. While the parasites predominate in internal organs, they occur in the skin macrophages as well, and it is probably from this site that the intermediate hosts, sandflies of the genus Phlebotomus, become infected. The parasites transform into the leptomonas stage, 14 to 20 μ long, and multiply in the midgut and foregut of the insect, which becomes infective after a week or more. The flagellates, injected by the bite of the fly into a new host, re-establish the vertebrate phase of the cycle.

L. donovani is easily cultivated in NNN and other mediums at 22° to 35° C., multiplying in the leptomonad stage as in the insect host. In tissue cultures of spleen from infected animals the nonflagellate stages

multiply abundantly.

Visceral leishmaniasis, or Kala-azar. kala-azar, is usually a chronic disease.96 Typically it begins, after an incubation period of one to four months (sometimes much longer), with a high temperature, followed by an irregular fever. The spleen and liver enlarge greatly with hyperplasia of the parasitized macrophage system. Wasting, emaciation, and edema are common. Dysentery often occurs as a result of heavy infection in the intestinal wall. The skin is typically dusky in hue, whence the name kala-azar, meaning "black fever." The skin is infected with the parasite but usually does not show lesions until months after systemic recovery when depigmented areas appear, later often becoming slightly raised papules. Anemia and leucopenia are characteristic. Adrenal insufficiency has been reported. Death is the rule in untreated cases, usually as a result of secondary infection.

IMMUNITY. Treated or spontaneously cured kala-azar is apparently followed by a lasting, solid immunity, for second infections are exceedingly rare. However, vaccines have failed to protect against or ameliorate

the disease.

DIAGNOSIS AND TREATMENT. Crucial

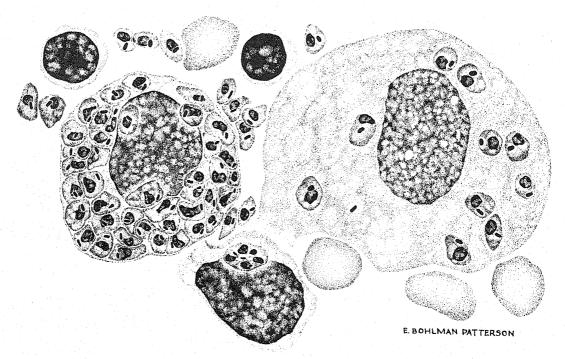


Figure 234. Leishmania donovani, free and in phagocytes, in Giemsa stained impression smear of spleen. Camera lucida; Approx. × 2000.

laboratory diagnosis of kala-azar may be obtained by the finding of parasites in biopsies of skin, spleen, liver, or bone marrow. Sternal bone marrow puncture is a reliable and safe procedure. Blood cultures may be positive. In addition, a group of nonspecific serological reactions has shown great value. These reactions depend on the fact that the serum euglobulin is greatly increased in amount in cases of kala-azar. In the formol-gel test (Napier's aldehyde reaction), positive serums form an opaque gel when formalin is added. In the antimony test a pentavalent antimonial drug causes a heavy flocculent precipitate in serums of cases. These tests are positive in over 80 per cent of cases and give false positives only occasionally with other diseases, especially schistosomiasis and malaria.

Several antimony compounds, particularly Neostibosan and Solustibosan, are effective in kala-azar. Successful treatment is often followed by dermal leishmaniasis, which may persist for several months. The drugs stilbamidine and pentamidine mentioned in connection with African sleeping sickness are also effective in kala-azar.

Epidemiology and control. Kala-azar has been reported in China, India, southern Russia, Mesopotamia, the Mediterranean littoral, southern Arabia, Equatorial Africa,

and parts of South and Central America. In Asia it is apparently a disease of man, though various lower animals, particularly dogs, hamsters, and mice, are susceptible. Infection may occur at any age but is most common in older children and young adults. In the Mediterranean area, however, it is primarily a disease of small children. Here dogs are commonly infected and apparently serve as the reservoir, since, unlike children, they show abundant parasites in the skin. The Mediterranean parasite is called by some authors L. infantum, and the South American form has been described as L. chagasi, but both are generally believed to belong to the species L. donovani.

Factors of seasonal and geographical distribution early pointed to sandflies as probable vectors, and it was soon shown that they were susceptible, developing heavy intestinal infections when fed on cases. In these flies the pharynx often becomes blocked with the simple flagellate stages, and in their vigorous efforts to feed, the "blocked" flies apparently are more likely to inject parasites.

The sandfly vectors belong to the genus Phlebotomus of the family of small flies, Psychodidae. The most important species are *Phlebotomus argentipes* in India, P.

chinensis in China, and P. major and P. perniciosus in the Mediterranean region. The vectors in other areas are as yet unknown. The adult flies are minute, night-biting insects. They fly weakly and travel very short distances but penetrate ordinary window-screening with ease. The larvae develop in loose damp soil or debris, mostly in cracks in walls, cliffs, caves, etc., the entire life cycle requiring one to two months.

The transmitting insects can be controlled by cleaning up potential breeding places or by spraying them with DDT. Treatment of human cases is also widely used for control

of the spread of kala-azar.

Leishmania tropica. L. tropica was discovered by Wright, in 1903, in an Armenian patient in Boston. Morphologically and culturally identical with L. donovani, it differs in infecting primarily the skin, where it proliferates in the macrophages of the subcutaneous tissue. In man, dogs, and wild rodents it produces large single or multiple ulcers, usually of exposed parts of the skin, known as oriental sore. These lesions appear after an incubation period of 10 days to several months. They increase to a diameter of 1 to 3 cm. and heal spontaneously after several months to leave a disfiguring scar. Permanent immunity follows infection, and deliberate induction of sores on unexposed parts of the body has long been practiced in the Middle East to avoid the disfigurement of exposed scars. Transmission can be effected by direct contact, fomites, and houseflies, but the principal spread is undoubtedly by sandflies in a manner similar to that in kala-azar. Oriental sore occurs in southern Asia, southern Russia, the Near East, equatorial Africa, and the Mediterranean region, the principal vectors being P. sergenti, P. papatasii, and, probably, P. caucasicus. Laboratory diagnosis is made by identification of the parasites in stained smears of scrapings from the lesions. Reliable diagnosis of oriental sore has been reported with an intradermal test. Since it is often very difficult to detect parasites in the lesions, such a procedure would be valuable. Local or systemic administration of antimony compounds such as Neostibosan is usually effective in treatment.

Leishmania braziliensis. Morphologically this species is indistinguishable from *L. tropica* and *L. donovani*, and its primary lesion closely resembles that of *L. tropica*. ⁵⁴ Superficial granular or moist ulcers may

occur at any skin site. In some endemic areas the parasite characteristically migrates to secondary foci, particularly mucocutaneous junctions, causing extensive destructive erosion of the nasopharynx, larynx, and associated structures, with facial disfigurement. Metastatic lesions occur with probable spread through the lymphatics and leishmanias have been recovered from the peripheral blood. Diagnosis and treatment are the same as used for the other leishmanias, by recovery and identification of the parasite in stained smears from lesions. An intradermal test, the Montenegro reaction, utilizing an antigen derived from cultured leishmanias is also of value. The disease is known by a variety of names throughout its range, including uta, espundia, chiclero ulcer, and mucocutaneous leishmaniasis and has a distribution ranging from Argentina north into The Yucatan peninsula of Mexico. In Central America, cutaneous leishmaniasis is a sylvatic disease, having reservoir hosts. such as small rodents and other forest animals. In South America the infection has been described from dogs.88

SPOROZOA

Malarial parasites. With the exception of an uncommon intestinal parasite, the only sporozoa infecting man are the malarial parasites. They were first recognized by Laveran in 1880, and the life cycle in human erythrocytes was described by Golgi. Manson's suggestions led Ross to the demonstration in 1898 of mosquito transmission of avian malaria, and Grassi, Bignami, and Bastianelli later in the same year showed the mechanism of spread of human malaria. Man harbors four species of malarial parasites, of which *Plasmodium vivax* will serve as an example. ¹³

Characteristics and life cycle. In fresh preparations of infected blood the parasites of *P. vivax* appear as clear areas in the erythrocytes. They contain yellow or brown granules of pigment, a digestion product of hemoglobin which has been identified microchemically as hematin. In blood films colored with stains such as Giemsa the parasites show blue cytoplasm and violet red nuclei.⁴⁷ The earliest stage in the erythrocyte, the *ring* stage, consists of a thin ring of cytoplasm with a nucleus at one side. The parasite grows, becoming an irregular uninucleate body containing several brown pig-

ment granules. This stage is known as the ameboid trophozoite. The parasitized cell has now enlarged somewhat and may show scattered throughout its cytoplasm minute red granules, "Schüffner's dots," which are apparently a result of injury to the cell. The parasite continues to grow, phagocytizing the erythrocyte hemoglobin which it digests, thereby accumulating more pigment. Eventually it nearly fills the erythrocyte, which is now about one and one-half times its normal diameter. The nucleus divides repeatedly until 12 to 24, usually about 16, nuclei are present. This is the schizont stage. Finally, in the *segmenter* stage, the cytoplasm divides, a portion surrounding each nucleus. The pigment is left in a dense clump, and the cell disintegrates to release the daughter cells, or merozoites, into the plasma. Here they invade fresh erythrocytes and repeat the cycle. The above process of growth and multiple fission is known as schizogony. It occupies, in P. vivax, about 48 hours, and the growth is regulated by the daily cycle of activity of the host, so that segmentation usually occurs at about the same time every other day.

After several schizogonic cycles a difference may be noted in the infection. Some ameboid stages, instead of becoming schizonts and undergoing asexual reproduction, develop into large uninucleate parasites with scattered pigment granules. These are the sexual stages. The female, or macrogametocyte, shows a compact, dark red nucleus and intense blue cytoplasm. The male, or *microgametocyte*, has a more diffuse, less deeply stained nucleus, and the cytoplasm is paler, often pinkish rather than blue. The gametocytes undergo no further development in man, eventually degenerating or being destroyed unless they are taken up by a susceptible mosquito.

In the stomach of the mosquito, gametes are produced. The macrogametocyte escapes from the erythrocyte and becomes a single macrogamete, corresponding to a metazoan ovum. The microgametocyte produces at its surface, by a process generally called "exflagellation," four to eight long, whip-like microgametes, counterparts of the spermatozoa of higher animals. One of these actively wriggling microgametes fertilizes a macrogamete. The resulting spherical zygote⁷² on the surface of the blood meal becomes entrapped between the contracting epithelial cells of the mosquito

stomach as the meal is digested. Growth results in an oöcyst which comes to rest on the outside of the stomach. Other authors believe that a fusiform oökinete is formed which actively penetrates the epithelial cells. The above process in the mosquito occupies one to two days. Nuclear multiplication occurs and the oocyst grows until a diameter of about 50 μ is attained. The oöcyst now contains many hundreds of nuclei. Each acquires a bit of cytoplasm and becomes a spindle-shaped body, about 8 μ long, the sporozoite. With the rupture of the oöcyst, these sporozoites scatter throughout the body of the mosquito. Many accumulate in the salivary glands, where they are injected into man by the biting mosquito. The complete development in the mosquito requires from one to two weeks: 25° C. is said to be the optimal temperature, no development occurring below 15° C. nor above 30° C.

For many years it was believed that the sporozoites entered erythrocytes to initiate the schizogonic cycle described above. Indirect evidence suggested that the parasites underwent a different type of development before invading the blood. This development was first discovered in a malarial infection of birds.⁷⁴ The sporozoites were found to enter fixed tissue cells where, as cryptozoites, they undergo a type of schizogony fundamentally like that in erythrocytes except that no pigment is produced. Segmentation produces merozoites which invade new macrophages and repeat the cycle. Some of the second generation merozoites enter erythrocytes to establish the blood schizogony. Others continue to reproduce in tissue cells as exoerythrocytic stages, probably throughout the whole course of infection, and may repeatedly give rise to new schizonts in erythrocytes. The cryptozoites and later exoerythrocytic stages are resistant to drugs effective against the blood parasites.14

Until 1948 neither cryptozoites nor later exoerythrocytic stages had been observed in mammalian malarias. They were observed first in monkey malaria and later in a human volunteer infected with enormous numbers of sporozoites of *P. vivax*. Large schizonts were found in the liver five to 10 days after sporozoite inoculation. Unlike those in avian malarias, which occur in macrophages, the cryptozoites in *P vivax* were said to be in hepatic cells. The parasites observed ap-

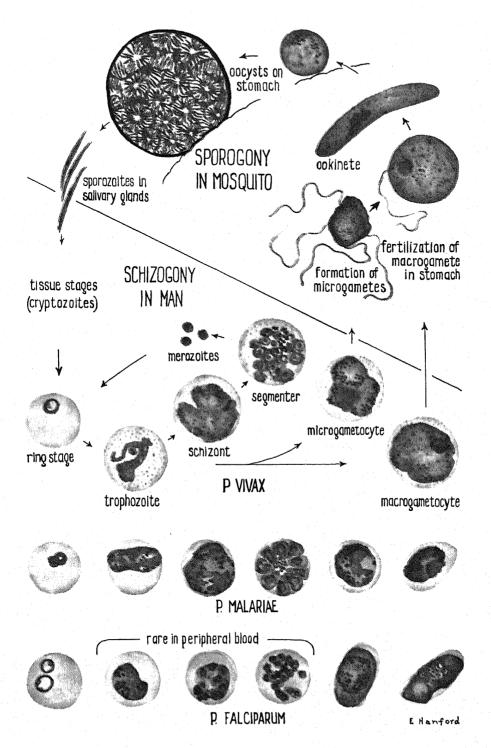


Figure 235. Life-cycle and comparative morphology of the malarial parasites of man. Oöcysts, × 600; remaining stages, × 2000. (Schizogonic stages redrawn from Huff: Manual of Medical Parasitology.)

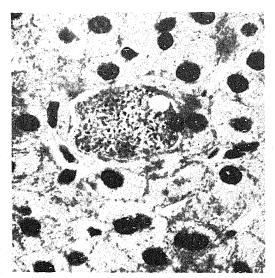


Figure 236. Cryptozoite of *Plasmodium vivax* in liver section. (Courtesy of Col. H. E. Shortt.)

pear to constitute a second generation of reproduction, but no organisms have been observed before the fifth day of infection.

In experimental infections with simian malaria, schizonts have been observed in the liver three and one-half months after infection, indicating persistence of the tissue parasites during and after the acute blood infection. Knowledge of various aspects of the exoerythrocytic cycle of plasmodia of all vertebrates is fully covered in a monograph by Bray¹⁴ and the more recent literature is covered by Eyles.³⁸

While much study is still required in this very important aspect of malaria, it is now possible to reconstruct in essence the development of infection in *P. vivax*. Sporozoites initiate a type of schizogony similar to that described above for avian malarias. In *P. vivax*, however, at least part of this cycle seems to be in cells other than macrophages. After about nine days some of the merozoites initiate the erythrocytic schizogony. Others apparently continue the exoerythrocytic infection, which can later give rise to new generations of erythrocytic parasites.

Limited success has been achieved in the artificial cultivation of malarial parasites. Geiman observed several generations of reproduction with *P. vivax* in erythrocytes in vitro. ⁵⁵ An avian plasmodium has since been maintained for 10 passages over a period of 18 days. Some growth has also occurred in a noncellular medium contain-

ing a lysate of erythrocytes.¹⁵⁰ A good deal of physiological information has come from these studies, but practical cultivation for routine work has not been obtained. Exoerythrocytic stages of avian malarias have been grown in tissue cultures of macrophages, and various cells derived from chick embryo heart, and chick meninges. Phase photocinematography of cultures are showing hitherto unsuspected morphological features of the exoerythrocytic schizonts and merozoites as well as providing information on motility and the mechanisms of cell invasion.⁷⁵

The life cycles and morphology of the other human malarial parasites in erythrocytes are fundamentally similar to those of P. vivax. P. malariae requires 72 hours for the completion of schizogony. The erythrocytic parasites show several differences of diagnostic value. No granules of the type of Schuffner's dots are seen in the infected cells. The cells are not enlarged during growth of the parasites. The older trophozoite is not ameboid like that of P. vivax but commonly exhibits a "band" form across the parasitized cell. The segmenter produces six to 14, usually eight, merozoites, arranged in 'a "rosette" form. The gametocytes are small, and the pigment typically occurs in abundant, large dark granules, the optimal temperature for sporogony in the mosquito is said to be about 22° C., and sporogony is slow, requiring three weeks or more.

P. falciparum, like P. malariae, causes no enlargement of the infected cells, but some parasitized cells show granules, Maurer's dots, which correspond to the Schüffner's dots of P. vivax. Schizogony results in six to 24 merozoites. The schizonts and segmenters typically accumulate in capillaries of internal organs, appearing in the peripheral blood only in very heavy infections. Normally, therefore, only ring stages and gametocytes are seen in blood films. Infection of erythrocytes by two or more rings is common and the rings often contain two nuclei. The gametocytes are distinctive, exhibiting a characteristic sausage shape which distorts or obliterates the infected cell. Because of their shape, they are commonly called "crescents." pigment is finely granular or amorphous in appearance and in the gametocytes it is typically concentrated about the nucleus. The optimal temperature for sporogony is said to be 29° C.

P. ovale is a rare species showing similarities to both P. vivax and P. malariae but characterized chiefly by the fact that the infected cell is often distorted into an oval. Its significance is not known, although many investigators consider it an aberrant type of P. vivax.

Malaria. Vivax malaria, or benign tertian malaria, is featured by typical chills and fever occurring at the time of segmentation of the peripheral blood parasites. These paroxysms begin with an acute, shaking chill while the temperature is rising. A "hot stage" occurs at the fever peak, the patient feeling unbearably hot and the oral temperature usually reaching 104° or 105° F. This gives way to a "sweating stage" during which the fever falls rapidly to normal or slightly below. Parasite products or red blood cell contents released at disruption of the infected cells are presumably responsible for the paroxysm. Paroxysms coincide, as stated above, with the time of parasite segmentation. Hence they occur every 48 hours if all the parasites reproduce on the same day. Often, however, distinct broods of parasites segment on alternate days. Vivax malaria commonly show quotidian (daily) chills for several days followed by the suppression of one brood of parasites with resultant tertian chills (every other day). Between paroxysms the patient feels and appears normal.

Significant anemia occurs commonly in vivax malaria but is rarely serious unless other factors are involved. A week or more after the beginning of an attack, particularly in children, the spleen usually enlarges and remains enlarged for two to six months after termination of the attack. The untreated attack typically lasts three to six weeks, often with temporary cessations of clinical activity. It is followed by a period of latency during which parasites cannot be found microscopically in the peripheral blood. During part of this time large transfusions of blood from the infected person fail to induce infection in recipients. This suggests that exoerythrocytic stages are responsible for maintenance of infection during latency. In many cases renewal of clinical activity, or relapse, occurs after two to 10 months of latency, the clinical picture resembling that in the primary attack. The infection usually dies out after two to three vears, and no further relapses occur.

Malaria due to P. malariae is known,

because of the 72-hour cycle of reproduction, as quartan malaria, the chills occurring every third day. It is basically similar to vivax malaria, though the paroxysms are often more severe. The incubation period is usually long, three weeks or more, and the period of clinical activity typically lasts for several months. Relapses are uncommon, but latent infection may persist for many years, as shown by the occasional development of quartan malaria in recipients of blood transfusions from persons who have not shown evidence of infection for 30 years or more.

Falciparum malaria is widely-known as malignant tertian or estivo-autumnal malaria. As noted above, P. falciparum is characterized by accumulation of schizogonic stages in internal organs. As a result, in addition to or instead of the typical paroxysms, which are quotidian or tertian, various local manifestations of the disease may be prominent. This is especially true in the tropics, where such "pernicious" malaria may follow a series of typical chills. The most common types are algid (cold) malaria, gastrointestinal manifestations, and cerebral malaria with coma and often death. These peculiarities of falciparum malaria make it the cause of most of the deaths attributable to human malarial infection and an important factor in many deaths traced to other diseases. Blackwater fever, a dangerous hemoglobinuria, is associated with falciparum malaria and is thought to be an occasional late effect of repeated infection. P. falciparum infections rarely relapse and rarely persist more than a year or two. It is believed that the exoerythrocytic stages do not persist after the cycle in erythrocytes has begun.

IMMUNITY. Malaria exhibits acquired immunity. After recovery from an acute attack, the individual is highly resistant to superinfection with a strain similar to that already present, but the resistance lasts for at most a few months after elimination of parasites from the body. This immunity depends on active phagocytosis and digestion of parasitized erythrocytes by the macrophages of spleen, liver, bone marrow, etc. That the parasitized cells are sensitized by antibodies is suggested by the fact that serum from recovered animals partially protects against infection. The immunity to superinfection is species- and strain-specific. During latency the parasites apparently

continue to reproduce by exoerythrocytic shizogony, sporadically producing blood parasites which are held in balance by the acquired immunity. Relapse occurs when this balance is disturbed, permitting the blood parasites to increase greatly in numbers. The factors are little known, though it has been shown that operations increasing blood sugar induce relapse in avian malaria.

Different strains of vivax malaria exhibit characteristic patterns of activity. Thus, an American strain typically relapsed about 10 months after the initial infection, while a strain from New Guinea usually relapsed within two months. Data such as these indicate that reactivation of latent malaria is at least partly determined by inherent cyclic properties of the parasites. The lesser susceptibility to, as well as the lessened severity of infection of, the negroid race to P. falciparum and P. vivax is cited as an example of racial immunity (Chap. Nine). Possession of the inherited sickle cell trait also confers a degree of immunity to P. falciparum as the erythrocytes are deficient with respect to the needs of the parasite. The maximum parasite count in those posessing the trait is much less than normal; the malaria mortality is less, resulting in the maintenance of a high population frequency of sickle cell anemia in some areas of P. falciparum endemicity. 104, 154

Partial inhibition of avian and monkey malarial infections has been achieved with killed vaccines. There is no evidence concerning human malaria, but, because of the nature of immunity to malaria, it seems unlikely that vaccination would be of practical value. Serum agglutination of parasitized cells or isolated parasites has been reported in experimental infections. It is species- or strain-specific. Complement-fixing antibodies occur in the serum and are group-specific, reacting with extracts of avian and simian malarial parasites as well as with those from man.

Diagnosis. Laboratory diagnosis of acute malaria rests on the finding of parasites in peripheral blood films. They are most easily identified in thin films stained with Giemsa, but, since parasites are often scanty in the peripheral blood, dehemoglobinized thick films, similarly stained, are widely used. The rapid staining method of Field⁴⁷ is of especial value for such preparations. The fact that parasite morphology is ab-

normal in thick films is offset by the much greater volume of blood which can be examined in a comparable time. In surveys the characteristic spleen enlargement is a nonspecific but highly suggestive indication of current or recent malarial infection. Because it usually persists for several months it affords a more stable index of the infection rate in an area than do clinical or parasitological surveys.

CHEMOTHERAPY. Treatment of malaria for centuries depended on the bark or extracts of the bark of cinchona trees. The main active principle in cinchona bark is the alkaloid quinine. Quinine neither prevents nor cures the natural infection but rapidly suppresses the number of blood parasites below the density necessary to produce symptoms. The synthetic drug quinacrine (Atabrine) has similar action, and in addition it can cure falciparum malaria. Chloroquine and Camoquin, developed in large-scale World War II research, have properties like those of quinacrine but are more effective.28 Paludrine, a product of British wartime research, is a very effective suppressive for vivax malaria and is both prophylactic and curative for P. falciparum infection. The distantly related Daraprim (pyrimethamine) has similar but stronger action. These two drugs suffer from the disadvantages that they act slowly and that the parasites may become resistant to them.

The above drugs taken continuously during exposure to vivax malaria act as suppressives, holding the infection down to subclinical levels. They do not prevent or cure the infection. *Primaquine*, however, destroys the exoerythrocytic stages and if given prior to invasion of the erythrocytes will prevent clinical malaria from occurring. It is also used in conjunction with drugs effective against the erythrocytic stages to cure established vivax infection, since elimination of the persistent exoerythrocytic stages is mandatory to prevent relapse.^{27, 117}

An exciting "repository" chemotherapeutic agent has been tested in a small series of men exposed to sporozoite and bloodinduced *P. vivax* infection. A single intramuscular injection of this dihydrotriazine metabolite of chlorguanide (Cl-501) protects against repeated infection for a minimum of seven months.^{29, 148}

As with other microorganisms, inheritable drug resistance can be induced in the plasmodia. 11,61 Development of pyrimeth-

amine resistance in both *P. vivax* and *P. falciparum*¹⁷² has been observed on a number of occasions, and particularly virulent strains of Chloroquine-resistant *P. falciparum* have been recovered in Colombia and Thailand.¹⁷³ The former retained sensitivity to pyrimethamine but the latter is resistant to pyrimethamine but Atabrine sensitive.

Epidemiology and control. Malaria is the most common infectious disease of man. occurring throughout the warmer regions of the world and extending well into the temperate zones to occupy a large part of the land area between 60° N. and 40° S. latitude. The principal factor in distribution of the infection is climate, which affects both the distribution and abundance of the mosquito hosts and the development of the parasite in the mosquito. The human malarial parasites are not found in lower animals. Man is, therefore, the only reservoir of infection. Racial immunity is known in the case of P. vivax. Negroes are less readily infected with most strains than whites and seldom show symptoms. Local populations with high natural immunity to P. falciparum have also been reported. Age resistance is not known, but in endemic regions clinical malaria may be rare in native adults who have been constantly reinfected with the local strains of parasites.

Barring rare congenital cases, blood transfusions, 12 and accidental transfers of blood, as by drug addicts sharing contaminated syringes, human malaria is transmitted exclusively by the bites of certain mosquitoes of the genus Anopheles. This genus, comprising some 200 species throughout the world, is readily distinguished from the common house mosquitoes of the genera Culex and Aedes by several characteristics. The adult generally rests at an angle to the surface, with proboscis, head, and body in a straight line, whereas the others rest parallel, with the head and proboscis turned down. The wings are usually spotted, those of other mosquitoes being unmarked. The palps of the female are as long as the proboscis, giving the impression of three long appendages from the head in addition to the antennae, whereas the other genera have short, barely noticeable palps. The eggs bear inflated floats and are laid singly on the water, whereas in Culex they are laid in rafts on the water and in Aedes they are deposited singly on damp surfaces. The

larvae of Anopheles lie flat at the surface of the water, feeding on floating particles, while those of other genera hang down in the water from an elongated breathing tube and usually feed on the bottom.

Only the female mosquitoes feed on blood, most Anopheles biting at dusk or in the night. The breeding places vary greatly in character, almost any type of water collection serving to support the larvae of one or more species of Anopheles. Most species, however, are quite specific in the types of water collections they choose. The principal malarial vector in the southeastern United States, Anopheles quadrimaculatus, breeds chiefly around the debris- and weed-covered edges of swamps, ponds, and sluggish streams. Important malarial vectors, however, are found in hill streams (A. minimus in Southern Asia), brackish marshes (A. atroparvus in Europe), small temporary pools (A. gambiae in Africa), water held in plants growing in the tops of trees (A. bellator in Trinidad), etc.

Not all species of Anopheles serve equally efficiently as vectors of malaria, about 75 being considered dangerous carriers in one or more regions. The chief determining factors are abundance, contact with man, and susceptibility. The last varies greatly among species and among populations within species. Little is known of the factors in human malaria, but simple genetic differences have been shown to determine susceptibility of some Culex mosquitoes to avian malaria. In most situations which have been adequately studied, contact with man is the principal factor in the importance of species of Anopheles. Several susceptible species are abundant in the southeastern United States, but in most of this area only one, A. quadrimaculatus, shows sufficient preference for human blood to serve as an important carrier. In Europe species (formerly lumped together as A. maculipennis) distinguishable only by morphology of the egg differ so greatly in their relative preferences for human and animal blood that some are dangerous vectors while others, equally susceptible, are insignificant in the spread of malaria.

Malaria control rests largely on the interruption of the mosquito phase of the life cycle. This may be accomplished by reduction in mosquito numbers or prevention of contact between mosquitoes and man. If mosquito reduction is to be attempted, the

Caluar and bowering males

differences in relative importance of different species, or even of the same species in different regions, require that the principal vectors in a given area be determined before control is attempted. Such determination is of value in avoiding waste of resources on unimportant mosquitoes. Furthermore, unplanned control efforts may completely miss the principal vectors and have even, on occasion, made matters worse by favoring the breeding of dangerous species. The "natural index" of infection, determined by examining stomachs and salivary glands of wild-caught female mosquitoes for occysts and sporozoites of malarial parasites, is the best guide to relative importance of a species. Unfortunately, the figure is often so low that dissection of large numbers of mosquitoes is required. In such cases expediency may justify indirect measures. Susceptibility may be determined by exposure of laboratory-reared mosquitoes to carriers and dissection of the fed females for stages of the parasite ("experimental index"). Contact with man may be measured by the location of resting mosquitoes (e.g., in houses as against stables) or by precipitin tests on fed mosquitoes to determine the type of blood they contain.

If control is to be applied to the breeding of mosquitoes, the habits of the important vectors in an area must be determined. Elimination of breeding places is the method of choice in many regions. Water collections may be destroyed by drainage or filling. Salinity may be controlled by tide-gates. Breeding around the margins of reservoirs may be eliminated by water-level fluctuations. Shade may be increased or decreased by control of vegetation. Streams may be cleared or periodically sluiced. Specific methods, it will be obvious, depend on local conditions and the habits of the mosquitoes involved. Several effective poisons (larvicides) are available for attack on the aquatic stages. Oils sprayed on the water surface are toxic to the eggs, larvae, and pupae. DDT in oil is extremely effective. A minor method of local value is the stocking of ponds and pools with top-feeding minnows. Gambusia or Lebistes, which eat mosquito larvae.

Attack on adult mosquitoes, formerly a secondary measure, is now the most important technique for malaria control. The insecticides, DDT, lindane and dieldrin, have given malariologists a weapon of tremendous value. Sprayed on walls, screens, etc., they

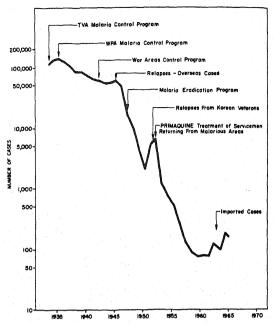


Figure 237. The incidence of malaria in the United States as indicated by the cases reported during the period 1933-1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare.)

kill adult mosquitoes which rest on these surfaces, even several months after application. For those species of Anopheles which enter houses to bite man, these insecticides have already demonstrated their value as an inexpensive and highly effective tool in malaria control. Malaria is no longer endemic in the United States and only sporadic cases of exotic origin are found today. The success of the control of malaria here and in several other areas, along with the development of better chemotherapeutic agents and economically feasible residual sprays, has led to the hope that world-wide malaria eradication will soon be an accomplished fact. 124 Detailed protocols and plans for this have been prepared. 112 However, in addition to the factors previously known, the problem has become further complicated by the development of plasmodia resistant to chemotherapeutic agents. This has become particularly evident in the strains of Plasmodium falciparum from various areas in southeast Asia resistant to chloroquine and other synthetic antimalarials.118 Another complication has been the development of insecticide resistance in anophelene mosquitoes. At the end of 1958 only three re-

sistant species were known and in the following year an additional 14 species of anophelene mosquitoes resistant to dieldrin and/or DDT were described.103 More recently another complicating factor has been found in a vivax-type malaria of macaques transmissible to man. Accidental transmission via mosquito has been recorded and further experiments indicate its ability to produce clinical malaria in man. Two strains of this parasite, Plasmodium cynomolgi, have been transmitted from man to man by mosquito. 10, 30, 135 The failure of control and eradication programs coupled with the continuing importance of malaria in military operations has caused a resurgence of basic research after an inactivity of almost 15 years. 130

Malarial parasites of lower animals. Malarial parasites fundamentally like those of man occur in apes, monkeys, bats, rodents, birds, and lizards. Since lower animal hosts are not readily infected with the human malarial parasites, the natural parasites of monkeys, birds, and, recently, rodents have been widely used experimentally. The species most widely studied are P. brasilianum, P. knowlesi, and P. cynomolgi of monkeys, P. cathemerium, P. relictum, P. gallinaceum, and P. lophurae of birds, and P. berghei of rats and mice. 53

INTESTINAL SPOROZOANS

Two species of coccidia, Isospora hominis and I. belli, are sporadic infectors of man although cosmopolitan in distribution. A resistant oöcyst is passed in the stools and infected individuals have slight to severe diarrhea. No information on the life cycle, therapy, or epidemiology is available and it is assumed that these species parasitize the epithelial cells of the small intestine as they do in the related species found in the dog and cat. The organisms have been described most frequently from the tropics and subtropics and have caused epidemics in military forces in the southwest Pacific islands during World War II. 45

PARASITES OF UNCERTAIN AFFINITIES

Toxoplasma gondii. Toxoplasma gondii was discovered in 1908 in rodents by Nicolle

and Manceaux in Africa and by Splendore in Brazil. The first clearly recognized human case of disease due to this parasite was reported by Wolf and Cowen in 1937. 128, 163

The organism is usually included with the sporozoa as a special group along with sarcocystis, with which it shares some characteristics. Some authors question their inclusion within the protozoa. Reproduction is asexual with cysts or pseudocysts containing naked trophozoites. Only a single host is required (monoxenous) and locomotion is by gliding or flexing of the cell, as no locomotor organelles are present.

Characteristics and life cycle. The trophozoite is a crescentic or oval body, 5 to 7 μ by 2 to 4 μ in size, staining blue with a reddish nucleus in Giemsa preparations as shown in the accompanying figure. It reproduces by binary fission in living cells. A great variety of cells are susceptible, but those chiefly infected are macrophages and other connective tissue cells, smooth and cardiac muscle, neurons, and microglia. While the parasites reproduce only in cells, they are often found free in body fluids.

In chronic infections they occur in a pseudocyst stage. This consists of a cluster of parasites resembling the trophozoites surrounded by an irregular wall. The origin of this wall is not known, but it may be the remains of a host cell in which the parasites proliferated. The pseudocysts are most abundant in the brain and skeletal muscle. They appear to be resting stages, more resistant than the reproductive forms.

Toxoplasma have not been cultivated in

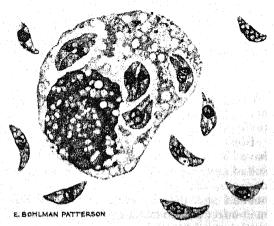


Figure 238. Toxoplasma gondii, free and in cell, from mouse brain smear. Giemsa, Camera lucida; approx. × 2000.

artificial mediums. They grow readily in tissue cultures of various types, including HeLa cells, and in chick embryos.

Toxoplasmosis. Although infection with T. gondii is very common in man, it rarely gives rise to acute disease. Most of the clinical cases occur in newborn infants infected congenitally during late fetal development. The active lesions predominate in the central nervous system and eyes, causing blindness, gross defects of the brain, and, not infrequently, death. Infection of adults is usually subclinical, but pneumonitis, enlargement of lymph nodes and spleen, fever, and a maculopapular rash may be exhibited. A severe chorio-retinitis may result from infection of the eyes. 113

Recovery is apparently accompanied by immunity to reinfection, although the pseudocysts, at least in lower animals, may persist for long periods in the tissues. Most experimental animals show solid immunity to reinfection. Monkeys and man exhibit a thermolabile antibody which protects rabbits against characteristic skin lesions resulting from the injection of toxoplasma.

Laboratory diagnosis requires isolation of the organisms or immunological tests. In neonatal cases it is usually possible to demonstrate the organisms by inoculation of laboratory animals. While all mammals and birds tested have been found susceptible, mice are preferred for isolation of the parasites because they are free from natural infection. Complement fixation may be used, but the most reliable immunological test is the dye inhibition reaction of Sabin and Feldman. This depends on the fact that toxoplasma in the peritoneal exudate of mice stain well with alkaline methylene blue in normal serum but not in immune serum. An accessory factor, present in fresh normal serum but destroyed by heat or by storage. is essential to the reaction. The test becomes positive two weeks or more after onset, reaching a titer of 1:256 or higher, and remains positive for many years. Fluorescent antibody methods have also been shown to be of diagnostic value.

Both acute and subacute toxoplasmosis have been treated with combinations of sulfadiazine and pyrimethamine. Treatment destroys the parasites in experimental animals and, though beneficial in some human infections, toxic side effects may occur with irreparable damage to the brain and eye.⁵⁰

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Epidemiology. Perhaps the most vexing question occupying investigators of toxoplasmosis is the method or methods of natural transmission. It is clear that congenital infection of human embryos can occur, but it is equally clear that other routes of infection exist. The parasite is extremely abundant in nature. Surveys with the dye test show that up to 60 per cent or more of human populations have been infected. Investigations in various parts of the United States have revealed past infection rates of 17 to 35 per cent, with rates of over 65 per cent in the age group above 40. A wide variety of wild and domestic birds and mammals show evidence of infection, often with high incidence. Human congenital infections have been associated with home environs in which mice and other vertebrates had abnormally high incidences of infection.³⁹ The pseudocysts in muscle are infective by mouth, and epidemiological evidence suggesting that infection might be acquired from undercooked pork has been reinforced by the finding of viable toxoplasma in samples of meat from 22 per cent of hogs from one slaughterhouse. However, orthodox Jews sometimes show evidence of a high incidence of infection, and some species of herbivorous animals are commonly infected, so it is obvious that pork or other meats are not the only important sources of infection. Toxoplasmosis should be regarded as one of the zoonoses in which man is only one of a relatively large number of natural hosts. Recently it has been demonstrated that parasitic nematodes (Toxocara) are capable of transmitting toxoplasma.78 The evidence suggests that the toxoplasma is transmitted within the nematode egg, and is liberated with or within the larval worm on hatching in the intestine of a warm-blooded host.

Sarcocystis. A single genus has been described with numerous species commonly found parasiting the muscles of mammals, birds, and reptiles. They are extremely common in ruminants, occasionally producing a severe pathology in sheep. Sarcocystis lindemanni, the species reported in a number of instances infecting man, is not known to produce detectable symptoms. The organisms are fusiform tubes, round in section, up to several centimeters in length and usually in striated muscle. These "Meischer's tubes" have a hyaline wall. Internally they are divided into compartments containing many thousands of sickle-shaped individ-

Manager and a second

uals 12 to 16 μ in length. Experimentally, infection may be accomplished by ingestion of infected meat.

Pneumocystis carinii. Infection with Pneumocystis is associated with highly contagious, epidemic, infantile, interstitial, plasma-cell pneumonia. The organisms are ovoid or crescent-shaped, 1 to 3 μ in diameter, and are commonly seen in rosettes of eight individuals within a membrane.

free or within phagocytes. The organism is considered to be a protozoan and the etiological agent of the disease, which in young infants frequently terminates in death. It has also been described from a wide variety of wild and domestic mammals. Although widely distributed, infection is but rarely diagnosed. Pentamidine and supportive measures have resulted in excellent decreases in mortality.⁷⁹

The Metazoa

All phyla of animals other than protozoa are commonly designated by the term Metazoa, and it will be convenient to mention certain general characteristics of the parasites of man, mostly known as "worms," which belong to this aggregate. They are usually macroscopic in size, ranging from about a millimeter to several meters in length. Their structure is generally complex. and their life cycles vary from the simple production of infective eggs or larvae to complex alternation of generations involving as many as three different hosts. An important general characteristic is that multiplication usually does not occur in the human body, so that infection does not increase in intensity in the absence of re-exposure. Until recently, true cultivation of worms parasitic in vertebrates had not been achieved. The great variety of metazoan parasites of man makes it impossible to discuss them extensively in a small space. Therefore, representative examples will be used wherever possible. For fuller discussions the reader is referred to the general texts cited earlier.

PLATYHELMINTHES

The phylum *Platyhelminthes*, or "flatworms," is differentiated from other phyla of animals by several characteristics. They are bilaterally symmetrical and composed of the three primitive germ layers of tissues—ectoderm, endoderm, and mesoderm. The body cavity is not lined with mesoderm, as in higher forms, but filled with a spongy mass of cells, the *parenchyma*. The digestive tract is absent or, if present, lacks an anus, solid wastes being regurgitated

through the mouth. There are three major classes—the Turbellaria, which are free-living forms or external parasites of aquatic animals, exemplified by Planaria; the Trematoda, or flukes, which are all parasitic; and the Cestoda, or tapeworms, also all parasitic.

TREMATODA

The human lung fluke, Paragonimus westermani, and the blood fluke, Schistosoma mansoni, will serve as examples of the trematodes.

Paragonimus westermani. The adult worm, found by Westerman in 1877 in the lungs of a tiger, was described by Kerbert in 1878. Nakagawa, Yokogawa, and others elucidated the life cycle. Several species have been described, differing but little in their morphology, and some authors believe that there is only one species parasitizing man, *P. westermani*. 1711

Characteristics and life cycle. The adult lung fluke is an ovoid reddish worm, 7 to 12 mm. long by 3 to 6 mm. in diameter, covered with a transparent cuticle which is studded with spines. The anatomy of a flattened specimen is shown in Figure 239. Two muscular suckers are present, one on the ventral surface, the acetabulum, the other at the anterior end, the anterior sucker, perforated by the mouth. The mouth opens into a muscular pharvnx, followed by a thin esophagus. This divides into two blind intestinal ceca extending down the sides of the body. The nervous system is simple, and no special sense organs are present. The excretory system consists of a bladder opening at the posterior end and receiving collecting tubules which extend throughout the body, terminating in characteristic

"flame cells." The circulatory system is rudimentary, consisting of indefinite channels through the parenchyma.

The adult fluke is hermaphroditic, both male and female reproductive systems occurring in the same individual. These systems are typically elaborate and, as in most flukes, are the most prominent structures of the body. In the female system a single ovary connects via an oviduct with the oötype, where the eggs are formed and receive their shells. In its course the oviduct receives the common vitelline duct, into which shell material comes from the branched vitellaria, glands occupying the sides of the dorsal surface of the body. The oviduct also has a diverticulum, the seminal receptacle, in which sperms are stored. An inconspicuous duct of unknown function, Laurer's canal, runs from the oviduct to an opening on the dorsal surface of the body. The oötype, surrounded by gland cells, Mehlis' gland, opens into the uterus, a long coiled tube which carries the completed eggs to the common genital pore near the acetabulum.

The male reproductive system consists of paired testes in the posterior part of the body, connecting by vasa efferentia with the vas deferens, which empties into the common genital pore along with the uterus. Part of the vas deferens is widened into a sperm reservoir, the seminal vesicle, and a more distal portion is the glandular prostatic region. The terminal portion forms a muscular copulatory organ, the cirrus.

The eggs produced by adult worms in the lung are coughed up and either escape in the sputum or are swallowed and passed in the feces. They are ovoid, averaging 60 by 90 μ in size, and show a removable cap. the operculum, at one end. The embryos are undeveloped at the time of escape from the host. They develop in from two to six weeks in water, where the eggs hatch by the opening of the operculum to release a free-swimming ciliated larva, the miracidium. This larva survives only a few hours unless it succeeds in penetrating the tissues of a suitable intermediate host. Various snails of the genera Melania and Pomatiopsis can serve as hosts. In the lymph spaces of the snail the miracidium becomes an irregular, thin-walled sac, the sporocyst, growing to a final length of about 0.4 mm. Cell masses within the sporocyst enlarge, developing in about one month into 12 or more rediae. These escape by rupture of the sporocyst and grow to a length of about 0.3 mm. They differ from the sporocyst principally in having a rudimentary digestive tract and a birth pore. By a reproductive process similar to that in the sporocyst the first generation redia produces 12 or more second generation rediae. These escape by way of the birth pore and grow to a length of about 0.5 mm. Within each second generation redia arise 20 or more tailed larvae. or cercariae. The cercaria is essentially a rudimentary adult worm. It differs principally in having a small tail and two types of penetration organs, a stylet in the mouth region and several gland cells, opening at the anterior end, which secrete histolytic enzymes. About three months after invasion of the snail by miracidia the cercariae escape and move about in the water, dying in one or two days unless they find a suitable second intermediate host, a crayfish or crab of any of various genera, especially Astacus and Potamon.

The cercaria penetrates the softer part of the integument of a crab, loses its tail and grows in the tissues into the *metacercaria*, a near-spherical body about 0.4 mm. in diameter enclosed in a cyst wall. The full development requires about one month, after which the metacercaria is infective when eaten by man. In the small intestine the cyst wall softens, releasing the metacercaria, which penetrates the wall of the small intestine and reaches the abdominal cavity within a few hours. It wanders rather aimlessly but usually passes through the diaphragm within a few days and invades the lung, where it becomes encapsulated by the tissues and grows to the adult stage. Considering this apparently random migration it is interesting to note that the pleural cysts commonly contain two or three adults. After about six weeks eggs may be found in the sputum.

Paragonimiasis. The adult worms in the lung, and particularly the eggs they produce, cause tissue destruction, inflammation, and hemorrhage. Local pneumonic processes with cough and bloody sputum are characteristic. As in most worm infections, the damage is roughtly proportional to the nul ber of organisms present. In severe case, weakness and even death may result from the extensive lung injury. Aberrant worms in other tissues, such as the brain, may produce local injury.

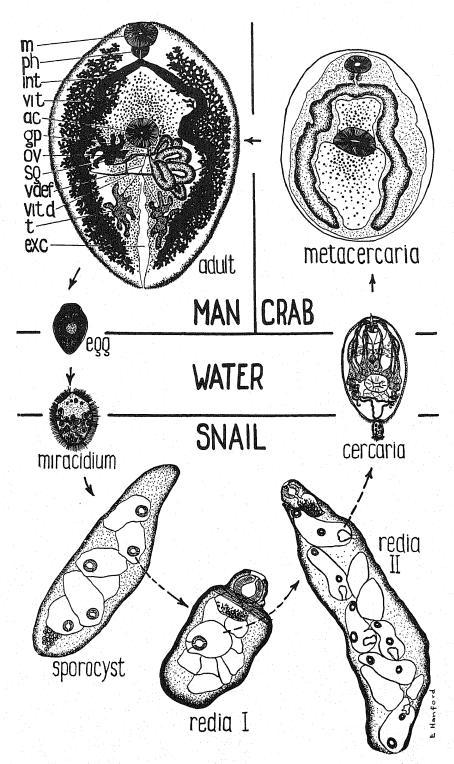


Figure 239. Life cycle and morphology of Paragonimus. m, mouth; ph, pharynx; int, intestinal cecum; vit, vitellaria; ac, acetabulum; gp, genital pore; ov, ovary; sg, oötype; vdef, vas deferens; vitd, vitelline duct; t, testis; exc, excretory bladder. Larval stages, \times 120; adult worm, \times 7. (Larval stages redrawn from Ameel, 1934.)

There is no evidence concerning acquired immunity to Paragonimus. Human infections have been shown to persist at least six years in the absence of re-exposure, but it is said that they often clear up after several years.

Diagnosis of lung fluke infection depends on the finding and identification of characteristic eggs in the sputum or feces. As in other trematode infections, the size and structure of the eggs are characteristic. 98 Other trematode eggs may occur in the feces, but only those of Paragonimus normally occur in the sputum. Specific immunodiagnosis is possible, using micro-Ouchterlony methods or immunoelectrophoretic patterns of human sera, reacted with Paragonimus antigens. 167

Epidemiology and control. Various mammals are susceptible to Paragonimus infection. Cats, dogs, mink, muskrats, and man are the usual natural hosts. Infection in lower animals is known in Asia, Africa, and North and South America, including the United States. Human infection is limited by food habits, occurring commonly only in the Far East. The metacercariae in infected crustacea are destroyed only by thorough cooking or considerable exposure to pickling sauces, and in the endemic areas

fresh-water crabs, raw or slightly pickled, are considered a delicacy. It should be emphasized that the asexual reproduction in snails is obligate and that only the metacercariae and possibly the cercariae are infective for mammals. Thus, direct transfer from mammal to mammal does not occur, and numerical increase of an individual infection is unknown in the absence of reexposure.

Chloroquine, in addition to its antimalarial activity, has been utilized with some success in a number of helminth infections inlcuding paragonimiasis. It is beneficial in early pulmonary infection but of dubious value in long-established infection or in ectopic cerebral parasitism. Good clinical results have also been reported with Bithionol. 168

Chemotherapy of human infections is useless for control, both because of its unreliability and because lower animals are an important reservoir. Destruction of snails has generally failed. The only effective control measure known is education to the dangers of eating improperly cooked freshwater crustacea. It should be noted here that while such modification of food habits is the best control method for all human trematode infections except the blood flukes,

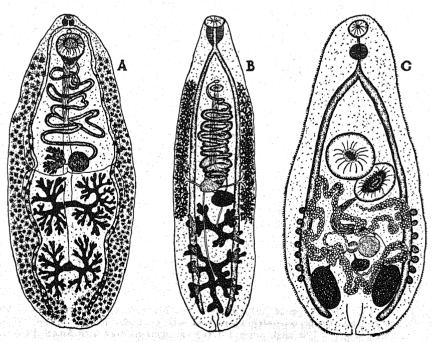


Figure 240. Trematode parasites of man. Note differences in magnification. A, Fasciolopsis buski, \times 3. B, Clonorchis sinensis, \times 5. C, Heterophyes heterophyes, \times 60.

established habits and prejudices often interpose difficulties in its execution. Yokogawa *et al.*¹⁷¹ have recently reviewed the status of knowledge of paragonimiasis.

Fasciolopsis buski. The large adults of this species attain a length of 7 cm. They are found attached to the mucosa of the small intestine of man and the pig. The infection is most common in China but occurs in other parts of Asia. The large eggs (average 140 by 80 μ) escape in the feces and develop in water, where the miracidia invade snails of the genera Segmentina and Planorbis. The development in the snail, like that of Paragonimus, comprises a sporocyst generation and two generations of rediae. The longtailed cercariae encyst as metacercariae on aquatic plants, particularly the water chestnuts. These plants, eaten raw or peeled with the teeth, convey the infection to man or the pig. Symptoms are generally related to the intensity of infection. They consist of intestinal disturbances and generalized edema. The latter has been attributed to toxemia but may be merely nutritional, resulting from the food-robbing of the host by the worms. Severe infections may cause death. As in many other intestinal worm infections, hexylresorcinol is therapeutically efficient. Control is achieved by treatment of human infections, proper sewage disposal, and education to the dangers of eating raw plants from contaminated water. The problem is complicated by the practice, common in endemic areas, of using human feces as fertilizer.

The closely related Fasciola hepatica infects the livers of sheep and cattle, causing "liver rot," and is of great economic importance. It is important also because it was the first trematode whose life cycle was described. Occasional human cases are known, usually contracted by eating fresh watercress to which metacercarial cysts are attached. Human infection is relatively frequent and clinically important in Cuba, Chile, southern France, and other scattered areas where sheep or cattle husbandry accompanies the raising of leafy vegetable crops commonly eaten uncooked. Fasciola gigantica and Fascioloides magna of herbivores also occasionally infect man. Another economically important parasite of the liver of herbivores, Dicrocoelium dendriticum, has been found rarely in man.

Heterophyes heterophyes. Heterophyes heterophyes is a small trematode, about 1.5 mm. long, of the intestine of man, cats, dogs, and other fish-eating mammals in Egypt, Asia Minor, and Asia. The eggs are small, averaging 25 by 16 μ . Metacercariae are ingested by man in insufficiently cooked or salted fish. The adult trematodes produce only minor gastrointestinal disturbances, but it is reported that the eggs are often deposited deep in the mucosa, whence they reach the general circulation and are localized in various distant tissues. The inflammation in these tissues, particularly the myocardium and brain, is said occasionally to cause serious symptoms or death.34 Several related species attack man. The most

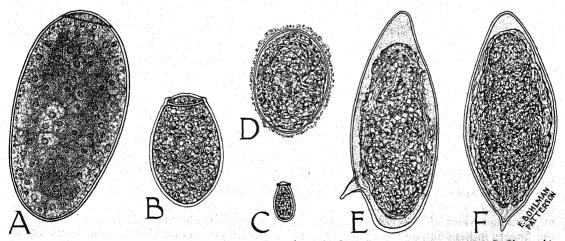


Figure 241. Trematode eggs in optical section. A, Fasciolopsis buski. B, Paragonimus westermani. C, Clonorchis sinensis. D, Schistosoma japonicum (note that the shell has the halo of debris usually seen when this egg is found). E. Schistosoma mansoni. F, Schistosoma haematobium. Camera lucida; approx. × 310.

important of these is *Metagonimus yoko-gawai*, which occurs in man and other fish-eating animals in the Balkans, Palestine, and Asia. A number of heterophyid flukes whose eggs cause extensive ectopic lesions have also been reported from man in the Philippines.²

Other intestinal trematodes. Several species of Echinostoma, Echinochasmus, and Euparyphium occasionally parasitize man. They are elongate trematodes with a ring of large spines on the head region. The human infection is acquired by ingestion of raw snails or mussels containing the metacercariae. Various mammals and birds are the normal hosts of the adult worms. Occasional human infections are also reported with Gastrodiscus hominis and Watsonius watsoni. Their life cycles are unknown, but since they chiefly parasitize herbivorous mammals it is presumed that the metacercariae are found on plants.

Clonorchis sinensis. The Chinese liver fluke, Clonorchis sinensis, is a thin, elongated trematode, 1 to 2 cm. in length. The adult worm inhabits the smaller bile ducts of man, cats, dogs, and other fish-eating mammals in China and Japan. The eggs, averaging 29 by 17 μ , pass out with the bile and escape in the feces. They are fully developed but do not hatch in the water. Upon ingestion by suitable snails, usually of the genera Parafossarulus and Bithynia, they hatch, and the miracidia become sporocysts in the lymph spaces. Rediae produced in the sporocyst give rise to long-tailed cercariae. These encyst as metacercariae in the tissues and on the skin and scales of various fresh-water fish. Metacercariae ingested by man hatch in the small intestine, and the young worms migrate up the bile ducts to develop into adults three to four weeks later. In rabbits experimentally infected, the young worms complete migration to the liver in hosts whose bile ducts have been ligated. Consequently, passage through the circulatory system to the liver or direct migration through the intestinal wall to the peritoneal cavity and into the liver surface may also occur.145 Thorough cooking (100° C. for 15 minutes) is necessary to destroy the metacercariae. Infection of man occurs by ingestion of the metacercariae, possibly in drinking water or by contamination of the fingers in handling infected fish, but usually by consumption of insufficiently pickled or cooked fish, a favorite article of diet in

the endemic regions. Hazards are increased in these areas by the practice of propagating fish for market in ponds fertilized with human feces.

Human infection is characterized by proliferation of the bile-duct epithelium and of the surrounding connective tissue. This results in liver cirrhosis, destroying liver parenchyma and obstructing portal blood flow. Intestinal disturbances, liver enlargement, and ascites are the common symptoms, severe infections resulting in death.

Although a variety of chemotherapeutic agents have been used for liver fluke infections of man, no reliable agent for the treatment of clonorchiasis has been available until recently. One of these used for the treatment of cattle and sheep liver flukes (1,4-bis-trichloromethylbenzol) has shown considerable promise, in its early trials. 169 Diagnosis depends on the identification of eggs obtained from feces or by duodenal intubation. There have been a number of efforts to develop practical immunodiagnostic methods for clonorchiasis. However, most of these cross-react with other trematode infections, such as paragonimiasis and schistosomiasis. A purified antigen prepared from adult clonorchis and consisting primarily of a polyglucose has been shown to have high specificity when used in a complement fixation test in this infection. 134 Control, as in the trematode infections discussed above, depends principally on education concerning avoidance of undercooked infected food, in this case fresh-water fish.

A closely related species, Opisthorchis viverrini, is of considerable importance in northern Thailand, where it infects 25 per cent of the population. 129 Opisthorchis felineus and O. tenuicollis infect fish-eating mammals and, less commonly, man in parts of Central Europe and Asia.

Human blood flukes: the schistosomes. The high incidence of infection of man with the several species of schistosomes, as well as the chronic debilitating disease produced, places these organisms among the world's most important infectious agents. The world incidence of infection is estimated to be greater than 4 per cent and some areas of endemicity are increasing in size. Ontrol measures and treatment of the individual are still inadequate. Bilharz, in 1852, first described trematodes in the veins of an Egyptian, and subsequently infection with the schistosomes was termed "bilharziasis"

in much of the medical literature. The worms are usually found in pairs in the portal venous system or in veins of the vesicle plexus with the female held in the ventrally grooved male gynecophoral canal. The split body of the male suggests the generic name for the group and, unlike the other trematodes mentioned, all blood flukes are dioecious. The adults attach to the walls of the blood vessels with their suckers and migrate to the smaller venules in copula for egg deposition. Occasionally they may develop in ectopic sites, probably being carried there by the circulation prior to attaining maturity. The blood flukes rely on a supply of carbohydrate and utilize glucose from the blood. 17, 160 In addition they ingest erythrocytes and have a globin-splitting enzyme. 149 Schistosoma mansoni is used here as an example of the group.

Morphology and life cycle. The paired adults of S. mansoni are normally found in the smaller mesenteric veins. The cylindrical female is 1 to 1.5 cm. in length by about 0.25 mm. in diameter. An anterior sucker surrounds the mouth and shortly behind it is a stalked acetabulum. The intestinal ceca unite before the middle of the body to form a single tube continuing to the posterior end. An oval ovary lies immediately anterior to the union of the intestinal ceca, and the uterus, containing eggs, extends forward to

open behind the acetabulum. The posterior half of the body is occupied by vitellaria. The male is shorter than the female, about 1 cm. long by about 1 mm. in diameter, its integument covered with coarse tubercles. The suckers and alimentary tract are similar to those of the female. The reproductive system consists of a cluster of eight or nine round testes in the anterior region emptying into a seminal vesicle, which opens to the outside just behind the ventral sucker.

S. mansoni has large eggs, averaging 150 by 65 μ , with no operculum but with a prominent spine at the side. They are deposited in the fine venules of the mesenteric veins in the wall of the intestine, where they lodge after the retreat of the ovipositing worms and the collapse of the vessels which had been distended by the worm body. The lateral spine aids in fixing the egg in the vessel and by a combination of events, probably including secretion of lytic substances by the developing embryo, the egg passes through the vessel wall. From the tissues the eggs continue migration through the mucosa and into the lumen of the intestine. They are mature at the time they leave the body in the feces. On dilution of the feces with water, the shell ruptures, releasing the miracidium. In suitable snails, Biomphalaria in Africa and Australorbis glabratus in the Western

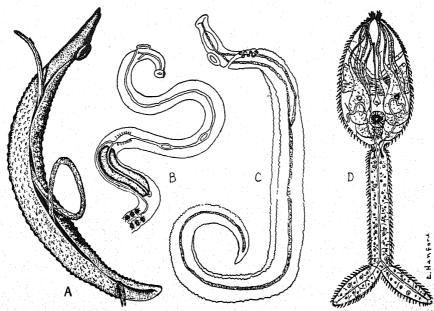


Figure 242. Schistosoma mansoni. Note differences in magnification. A, adult worms, \times 8. B, anterior end of female worm, \times 20. C, adult male, \times 10. D, cercaria, \times 180. (A and C redrawn from Manson-Bahr. D. redrawn from Cort.)

Hemisphere, the miracidium penetrates and two generations of sporocysts are produced resulting, after about six weeks, in the production of cercariae with long, forked tails. Escaping into the water the cercariae swim actively or rest at the surface, dying in about three days if they do not reach a susceptible final host. Coming in contact with human skin they attach and actively penetrate the skin. The epidermis is passed quickly and the cercariae shed their tails and may reach lymph vessels in the dermis within 15 minutes or less. This rapid invasion of the unbroken host skin is accomplished through use of cercarial enzymes as well as by muscular activity. A major feature of cercarial morphology is the possession of sets of large unicellular "penetration glands" which are the source of mucoid secretions, collagenaselike proteases, and mucopolysaccharidases, all presumed to be of importance in invasiveness of these parasites. 92, 141 The parasite is carried via lymph and blood to the portal veins in the liver, where maturation occurs and the adult forms mate and migrate distally in the portal system to the mesenteric veins. Four to eight weeks after infection the sexually mature worms begin egg production. The infection may persist for over 25 years without re-exposure.

The other species of human blood flukes differ from S. mansoni in size, details of morphology, egg structure, location in the definitive hosts, types of snail hosts, and geographical distribution. S. haematobium adults occur predominantly in the venules of the urinary bladder wall, the eggs escaping in the urine. The eggs are large, 150 by 60 μ , with a terminal spine. The male, 1 to 1.5 cm. long, is covered with fine tuberculations and has four large testes. The female is about 2 cm. long. Snails of the genera Physopsis and Bulinus serve as intermediate hosts.

S. japonicum occurs in the venules of the intestine. The eggs average 80 by 65 μ , with a small lateral knob rather than a spine. They escape in the feces, the miracidium invading snails of the genus Onocomelania. The male is 1 to 2 cm. long, with a smooth integument, and has seven or eight testes. The female is 1 to 2.5 cm. long.

Schistosomiasis. The disease produced by the schistosomes varies with the infecting species, the number of adult worms present, and with the duration of infection. The pathology may conveniently be divided into the incubation period, from skin penetration to egg deposition, the acute stage, and the chronic stage. At the inception of the incubation period, skin penetration may cause a mild transient dermatitis. In the forms normally parasitizing man this is less severe than that produced in man by the penetration of cercariae normally parasitizing other vertebrates (see below). The larvae in passing through the lung may cause an eosinophilic infiltration and while developing in the portal system, parasite metabolic products cause a toxic hepatitis often accompanied by irregularly fluctuating fever and allergic manifestation such as urticaria. This is followed by the acute stage in which eggs are actively deposited in the tissues and are extruded into the lumina of the intestine or bladder. During this period trauma may produce intestinal hemorrhage and pseudodysentery in S. mansoni and S. japonicum infections. Hematuria is characteristic of this stage of S. haematobium infections. 56, 114 Even in these early stages numerous eggs may escape into the circulation rather than migrating through tissues to organ lumina. These embolic eggs lodge in numbers in the liver, where they become enclosed in small granulomata (pseudotubercles) and in S. haematobium and S. *japonicum*, numbers commonly pass to the lung. In many instances in S. japonicum, relatively large numbers reach the brain, producing the symptomatology of cerebral damage. The chronic stage is due to the host's reaction to continuous egg-produced tissue damage. In S. mansoni the wall of the large intestine becomes thickened with scar tissue and rectal polyps of connective tissue with eggs may be extensive. Similarly, impairment of the small intestine and thickening occur with S. japonicum, and in both, cirrhosis of the liver is a most important sequel. With S. haematobium the wall of the urinary bladder is greatly thickened with fibrous connective tissue and the urethral lumen is commonly reduced. 69 The external genitalia are also commonly involved. Carcinomas of the rectum, liver, and urinary bladder are often observed and in some areas a high correlation between these malignancies and schistosomiasis is claimed.

Concomitant with the liver damage found in S. mansoni and S. japonicum infections, circulatory impairment produces portal hypertension, splenic enlargement, esophageal varices, and ascites.^{5, 52} As briefly summarized above, the pathology produced varies with time and also with the differing site of

egg deposition by the different species of adult worm. Another important species difference in the disease is the fact that the female S. japonicum produces 10 times the number of eggs per day as the S. mansoni female. Egg production of the female S. haematobium is believed to be intermediate between these.

IMMUNITY. Direct evidence of functional immunity to schistosomes in man is lacking. Children and young adults commonly have severe infections which may terminate fatally. Some infected adults are found without symptoms of progressive disease under conditions where it may be assumed that they have periodic exposure to reinfection with S. japonicum or S. mansoni. Animals cured of initial infection show some resistance to reinfection and, in monkeys, some individuals surviving initial infection are strongly immune to massive reinfection. 158 Recent evidence indicates promise that immunization by vaccination may be possible as rhesus monkeys inoculated with a nonhuman (Formosan) strain of S. japonicum exhibited considerable resistance to challenge with the human strain (Japanese).⁷³ In addition, mice inoculated with cercariae of S. mansoni that had been exposed to 60 cobalt radiation developed a relative immunity to reinfection with nonirradiated cercariae. 156

A wide variety of serological evidence of antibody production in schistosome infection exists and in individual human sera circumoval precipitins, miracidial agglutinins, cercarial agglutinins, complement-fixing, and other antibodies can be demonstrated.⁸² In addition, serum proteins inhibiting cercarial penetration enzymes are also produced.⁹² To date these serological phenomena cannot be correlated with the degree of functional or protective immunity of the individual.

DIAGNOSIS. Laboratory diagnosis is based on the finding of characteristic eggs in the feces, urine, rectal biopsy, or rarely, liver biopsy. In very early or in late chronic stages the eggs may not readily be found, and multiple examinations plus concentration techniques must be routine for diagnosis of individual infections. Immunological techniques are also valuable both for the individual diagnosis and for epidemiological surveys. These include the production of precipitate about viable or lyophilized eggs (circumoval precipitin reaction), high titer hemagglutination of erythrocytes sensitized with adult antigens, intradermal reaction to adult antigens, binding of fluorescent antibody by preserved cercariae, ¹³¹ and a variety of other methods including complementfixation and gel precipitation techniques.⁷⁴ Some of the reactions are group-specific, persist after cure, or are negative in a high percentage of the lower age group. The circumoval, fluorescent antibody, and intradermal tests are of practical value if their limitations are understood.

THERAPY. The most effective treatment of all three species is the intravenous administration of potassium or sodium antimony tartrate, which properly administered will provide a high percentage of cures (84 per cent in S. japonicum). 16, 18 This tartar emetic requires slow administration on alternate days over a one-month period, is painful, and not practical for mass treatment. Consequently Fuadin (Stibophen), an intramuscularly administered antimonial, is a favored drug in many of the endemic areas. Characteristically, it sterilizes temporarily the female schistosome, causing cessation of egg-laying and of symptoms referable to this damage. Properly used, cures may be effected. However, the cure rate is low, since a proper course of therapy is seldom followed and the female worms are believed to recover. The mechanism of action of the trivalent antimonials is an interference with one of the enzymes (phosphofructokinase) of the helminth carbohydrate metabolism.¹⁷ Miracil D, an orally administered drug, has been shown to be of some value in the treatment of S. haematobium infections and is of particular value in small children.

Epidemiology and control. The chief means of infection is by contact of the skin with water containing the cercariae. This occurs especially in the working of rice fields fertilized with human feces, but also in bathing and chance contact. Infection may be acquired from drinking water and rarely as an intra-uterine infection. Man is the only important reservoir of S. mansoni and S. haematobium infection although both occur naturally in monkeys and rodent infections of S. mansoni have been described. The disease is for these two species perpetuated by the promiscuous discharge of urine and feces by man or their deliberate use as fertilizer in the cultivation of rice, sugar cane, and similar crops. Infection of streams and irrigation canals used for ablution and the washing of clothes is important in maintenance of the infection. In contrast to these forms, S.

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japonicum has numerous reservoir hosts among domestic and wild mammals which pass eggs in feces and may maintain the infection in man's absence. S. mansoni is widely distributed throughout Africa whereever suitable snail intermediate hosts are found. The delta of the Nile is an endemic area of primary importance; it is common throughout tropical Africa to South Africa and in much of its range coexists with S. haematobium. Importation of S. mansoni with slaves established the disease in Brazil, Venezuela, Surinam, and the West Indies including Puerto Rico. In these areas it remains of major importance and the world incidence approaches 40 million. The present distribution of S. haematobium not only includes all of Africa suitable for its development but also parts of Arabia, Palestine, Lebanon, Syria, small endemic areas in Portugal, and India. S. japonicum is limited to the Far East with important endemic areas, in the Philippines, China, the Celebes, and Japan. A number of small foci have been found in Thailand and Laos, and a strain primarily parasitizing dogs and rats is found in Taiwan.

Control of schistosomiasis depends on destruction of the snail intermediate hosts, prevention of access of eggs to water containing snails, or avoiding contact with cercaria-containing waters. Some limited success has been obtained in snail control using copper sulphate or sodium pentachlorophenate as molluscicides. 101 Periodic drying of irrigation ditches is of some value for the control of snails transmitting S. mansoni and S. haematobium but of little value in S. japonicum control as the snail host is operculate and resists desiccation. As it does not survive well in ponds or in welldrained areas, ponding and drainage are utilized in control. In limited areas having special characteristics the introduction of a competitor, the snail Marisa, has eliminated Australorbis, the host of S. mansoni. 119 Safe sewage disposal and increased sanitation has not met with outstanding success as a control measure, since the customs of the people as well as the economics of the endemic areas operate against these methods. Avoidance of contact with infected water is difficult in some areas, since it is also associated with religious habits of ablution; in other areas only infected waters are available for drinking and general cleansing, and it has not been economically possible to provide safe water supplies.

Other schistosome species. A number of species commonly parasitizing ungulates of Africa and India, including S. bovis and S. spindale, have been reported as occurring in man. Several hundred cases of S. intercalatum in man have been reported from tropical Africa. This form has a terminal-spined egg slightly larger than that of S. haematobium. The eggs are passed in the feces rather than in the urine.

Schistosome dermatitis. Several species of schistosomes, especially in the genus Trichobilharzia, may invade the skin of man to cause severe allergic dermatitis. These worms, which normally parasitize birds or lower mammals, apparently cannot mature in man. While dermatitis-producing schistosomes have been recorded from scattered areas throughout the world, excepting Africa, they are prominent only in parts of Europe and North America. Certain lakes in the north central states, particularly in Michigan and Wisconsin, were formerly notorious for the "swimmer's itch" contracted by bathers.31 Although snail destruction with copper salts has successfully controlled the infection in some of these areas, this type of cercarial dermatitis continues to be a serious problem over much of this area. The dermatitis can be treated with anti-allergic ointments. One of the parasites producing schistosome dermatitis, Schistosomatium douthitti, is of especial interest because it is easily studied in laboratory mice. Recently, avian schistosomes causing a marine dermatitis have been described from salt-water beaches of Hawaii, California, and the eastern United States.²⁶

CESTODA

The cestoda, or tapeworms, generally consist of a colony-like chain of flattened segments, each of which is a semi-independent unit with a complete set of reproductive organs. A scolex at the anterior end of the chain serves to anchor the worm in place, and the nervous and excretory systems are shared by the whole organism. There is no alimentary tract, soluble food substances being absorbed through the body surface. All tapeworms are parasitic, the adults usually in the alimentary tract of the definitive host and the larval stages in the tissues of an intermediate host.162 Two orders of tapeworms, the Pseudophyllidea and Cyclophyllidea, contain human parasites. The Pseudophyllidea are discussed later (see

Diphyllobothrium latum). Most of the tapeworms of man belong to the Cyclophyllidea, of which Taenia solium will serve as an example.

Taenia solium. The pork tapeworm, Taenia solium, was known in ancient times. Küchenmeister, in 1855, first suspected its relationship to "bladderworms" in pork and demonstrated their transformation into the adult worms in the intestine of a condemned criminal.

Characteristics and life cycle. In the intestine of man the adult T. solium attains a length of 2 to 3 meters. Buried in the intestinal mucosa of the host is the scolex, or "head," a rounded cubical organ about 1 mm. in diameter bearing four large, cup-like suckers and a pad, the rostellum, with 20 to 35 hooks. Behind the scolex is a thin neck which merges into a region of proliferation of segments, or proglottids. As new segments are produced, the older ones are pushed back, developing sexually, until near the middle of the worm mature proglottids are found. They average 0.5 cm. square. The genital pore occurs on either side of the proglottid, alternating irregularly from segment to segment. The male reproductive system consists of small follicular testes scattered throughout the dorsal part of the segment, emptying through vasa efferentia into a coiled vas deferens which ends in the muscular copulatory organ, or cirrus, at the genital pore. In the female system the vagina extends from the genital pore to the oötype in the posterior central part of the segment. On either side of the ootype are ovaries and behind it the vitellaria. A blind uterus extends forward from the ootype.

As the segments move toward the posterior end of the worm, the uterus fills with eggs, becoming branched to fill most of the segment in the gravid proglottid, which is about 0.5 by 1 cm. in size. In T. solium there are about 10 branches on each side of the uterus. The gravid segments become detached singly or in small groups and pass out with the feces. Before or after escape from the body they disrupt, releasing large numbers of eggs. These eggs, averaging 35 μ in diameter, are nearly spherical and provided with a thick "shell" of characteristic porous structure. Within the shell is seen the onchosphere, an embryo bearing six hooks.

Ingested by a suitable intermediate host, the eggs hatch in the intestine and the embryos penetrate the intestinal wall to enter the blood or lymph. They are carried to various parts of the body, developing in the tissues in about two months to the infective stage. Infection is most intense in the muscles, but other tissues also contain the parasites. This stage, a cysticercus, consists of a scolex like that of the adult, a short neck, and a fluid-filled sac about 1 cm. in diameter. Because of the sac, this larval stage is commonly called a "bladderworm." In the tissues the scolex is invaginated into the bladder. In addition to the pig, other animals may harbor the cysticerci. Among them is man, in whom they occur in various tissues including the brain.

Man acquires the adult tapeworm by ingestion of undercooked pork containing the cysticerci. In the intestine the bladder is digested away, the scolex attaches to the intestinal wall, and the adult worm develops

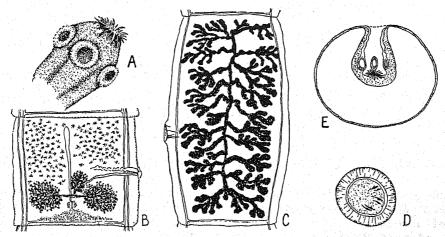


Figure 243. Taenia solium. A, scolex, \times 20. B, mature proglottid, \times 5. C, gravid proglottid, \times 5. D, egg, \times 500. E. cysticercus, \times 5. (Adapted from various sources.)

by growth from the neck region. By two months after infection gravid proglottids containing eggs are being passed in the feces. Infection may last for many years.

The human disease. Adult pork tapeworms usually occur singly in the intestine of man. They often cause no noticeable symptoms, though general intestinal discomfort may occur, and in children nervous disturbances are sometimes seen. The ravenous appetite popularly associated with tapeworm infections is actually uncommon, loss of appetite being more frequently observed. In contrast with infection by the adult worm, tissue infection with the cysticerci is dangerous. The larvae act like benign tumors; growth is slow and terminates naturally with full development. The injury results from pressure, and its seriousness, therefore, depends on the location of the larvae. In most muscles and connective tissues they are of no consequence, but larvae in the eye may affect vision. In the brain they may give rise to epilepsy or other manifestations of local pressure.

There is no evidence of acquired *immunity* to *T. solium* in man, though it has been suggested that the rarity of multiple infections in the intestine indicates immunity to superinfection. In individuals harboring the cysticerci, complement-fixing and precipitating antibodies are detectable. They react with extracts of other tapeworms as well as *T. solium*.

Laboratory diagnosis of intestinal infection with T. solium is based on recovery of eggs or gravid proglottids from the feces. The eggs are not distinguishable from those of the beef tapeworm (see below), but the gravid segments can be identified by the smaller number of uterine branches, about 10 on each side as against more than 15 in the beef tapeworm. Diagnosis of the cysticercus infection is rarely made before operation or necropsy, but the immunological reactions mentioned above have been used. Quinacrine hydrochloride (Atabrine) is used in treatment by tablet or sometimes by duodenal intubation. Extract of male fern (Oleoresin of Aspidium), an old therapeutic agent, is still used in some instances. There is no treatment known for the larval infection except surgery.

Epidemiology and control. The larvae of T. solium occur most commonly in pigs but are found also in man, monkeys, sheep, camels, and dogs. Before 1850 in Berlin 2 per cent of human autopsies showed the cysticerci, but the incidence is now much lower. Pigs acquire the infection by contamination of their food or water with human feces. In some areas of the tropics and the Far East pigs, because of their coprophagic habits, are responsible for the relative cleanliness around small rural villages. Larval infections in man are incurred chiefly by contamination of food, water, or fingers with eggs from human feces, but it is also possible that in the intestine of an individual harboring the adult worm the eggs may hatch without reaching the outside. Whether or not eggs must leave the body before they are infective, a person with the adult worm in his intestine is a constant hazard to himself as well as to others. Man, the only known host for the adult tapeworm, acquires the infection by ingestion of undercooked pork, which often contains large numbers of larvae. Freezing and thorough cooking kill the larvae, but ordinary pickling and smoking are ineffective. Government meat inspection reveals most infected carcasses and has been the most effective single control measure. Other control methods are directed at the prevention of contact between pigs and human feces.

The beef tapeworm, Taenia saginata. Taenia saginata, resembles T. solium in morphology and life cycle, but several differences are noteworthy. The scolex is "unarmed," lacking the hooked rostellum of T. solium. The adult worm is usually about 5 meters long, but occasionally is much longer. The gravid proglottids are distinguished from those of T. solium by a larger number of uterine branches, usually 15 to 20 on each side. Man is the only known host of the adult. The cysticerci develop in cattle and other ruminants, human infection resulting chiefly from consumption of undercooked beef. Rare cases of human infection with the larvae have been claimed, but it is probable that they were abnormal cysticerci of T. solium.

Echinococcus granulosus. The adult of Echinococcus granulosus inhabits the intestines of dogs and related species. It is a minute worm 0.25 to 0.5 cm. in length, consisting of an "armed" scolex and three proglottids, one immature, one mature, and one gravid. Large numbers of the adults may occur in the intestine of an infected dog.

The natural intermediate hosts are sheep, cattle, and other ruminants, but a wide va-

riety of animals are susceptible, including man, in whom the larva causes a serious disease. This larva is markedly different from those of the beef and pork tapeworms discussed above. In the viscera of an infected animal it attains a diameter of about 1 cm. after five to six months. It is infective after eight months or more but continues to grow, often, after several years, reaching a diameter of more than 20 cm. The larva, known as a "hydatid cyst," is a spherical, fluid-filled sac composed of a thick cuticular wall with a thin germinative epithelium on its inside surface. From this germinative epithelium are formed two types of structures. Buds may appear and grow into stalked vesicles, brood capsules, on the inner surface of which stalked cysticercus-like "scolices" are produced. These brood capsules may become detached as daughter cysts, enlarge, and produce brood capsules and scolices within themselves. In the large cysts there may thus be produced many thousand infective scolices, each of which can develop into an adult worm if the cyst is eaten by a dog feeding on the viscera of an infected animal. Thus E. granulosus differs from T. solium chiefly in the fact that multiplication occurs in the larval as well as the adult stage. The predominant site of larval infection is in the liver. The lung is next in importance, and cysts may occur also in practically every other organ.

Hydatid disease in man. Echinococcosis, or hydatid disease, is characterized by two general types of manifestations. First, hypersensitivity develops to components of the hydatid fluid, and accidental rupture of a cyst may cause serious, even fatal, reactions. Second, and most important, the growing cyst acts like a tumor, the injury resulting from pressure effects and depending on the localization of the cyst. Rupture of a cyst may give rise to new cysts produced from scolices, daughter cysts, or fragments of germinative epithelium. Osseous cysts, occurring in bone, may weaken the bone by erosion of its structure.

There is no information available concerning acquired *immunity* to the larval infection in man, though in immunized sheep the cysts develop somewhat abnormally. Dogs injected with antigens derived from the larvae are immune to infection with the adult worm.

Laboratory diagnosis is usually based on immunological reactions. The precipitin test

and the Casoni intradermal reaction are both useful but remain positive after removal of the cysts. The most reliable test is the complement-fixation reaction, utilizing merthiolate-preserved cyst fluid as an antigen.

Epidemiology and control. The normal life cycle of E. granulosus involves dogs and sheep or cattle, man being an accidental host of the larva. For this reason, the infection is most common in the great grazing regions of the world, particularly Australia and New Zealand, North and South Africa, Iceland, and southern South America. The incidence in cattle and sheep occasionally exceeds 50 per cent. Various wild mammals may also be involved, among them moose, reindeer, and wolves in northern North America. Transmission of Echinococcus eggs to food by sarcophagic flies from dog feces has been experimentally demonstrated and may be presumed to occur naturally.

Control of the infection in domestic animals depends chiefly on preventing dogs from eating offal. Possibly infected waste material should be buried or burned. Prophylaxis of human infection requires measures to reduce the chance of contamination of human food and water by feces of dogs.

Echinococcus multilocularis. The adult of *E. multilocularis* differs from *E. granulosus* only in fine morphological detail. The larva, however, develops differently from the discrete, spherical cyst produced by the latter, forming an *alveolar cyst* which spreads malignantly as a foaming mass of minute, budding globules, each containing, in the normal hosts, a few scolices. The liver is usually infected, and it is extensively destroyed by the exuberant growth of the larval tissue. ¹⁵⁷

E. multilocularis has been found in central Europe and the Aleutian Islands but probably also occurs in northern Asia. The adult hosts are foxes, and the larvae normally parasitize wild rodents. Sporadic larval infections occur in man, causing usually fatal destruction of liver tissue.

Alveolar hydatid was known in man for nearly a century before, in 1951, Rausch and Schiller recognized it as a distinct species. It had previously been considered an abnormal form of *E. granulosus*. The name *E. alveolaris* has also been applied to it.

Hymenolepis nana. The human dwarf tapeworm, Hymenolepis nana, is a small worm, 1 to 4 cm. in length, with mature proglottids 0.5 to 1 mm. in breadth. The scolex is similar to that of T. solium but smaller,

about 0.25 mm. in diameter. The mature proglottids are markedly different in appearance from those of *T. solium*, the width being more than four times the length. Three large testes occur in each segment. The uterus in the gravid segments is a large, irregular sac. The gravid segments are usually destroyed in the intestine, releasing the eggs, which are found in the feces.

H. nana is unique among human tapeworms in not requiring an intermediate host. Ingested eggs hatch in the intestine of man to release an embryo which invades the tissues of an intestinal villus. Here, in about four days, it develops into a cysticercoid, a larva with a small bladder and a solid tail. This larva breaks out of the intestinal wall. attaches to the mucosa, and develops into the adult worm. Eggs are found in the feces about two weeks after infection. It is reported that several types of insects can serve as intermediate hosts, but they are probably unimportant in human spread of the parasite. Large numbers of worms are commonly found in an infected individual, and they may give rise to intestinal and nervous disturbances in children. Epileptiform convulsions may occur. Ouinacrine hydrochloride (Atabrine) and extract of male fern are useful drugs for elimination of the worms.

H. nana occurs naturally in man, monkeys, and rodents. Although the form in rodents has been separated as a distinct species, H. fraterna, it is generally considered a variety of H. nana. The parasite is cosmopolitan. It is much the most common tapeworm of man in the United States, especially prevalent in children, but occurring in all age groups. Age resistance has not been shown in man but is experimentally demonstrable in rats and mice.

The lack of an intermediate host permits direct spread from man to man. It is not known whether the eggs can hatch without having reached the outside. However, an infected individual is particularly liable to reinfection from fingers contaminated with his own feces.

The closely related *H. diminuta* of rats and mice is transmitted by several species of insect intermediate hosts. Occasional human infections have been reported, mostly in children.

Tapeworms of lower animals. Species of the genus Multiceps exhibit the adult stage in dogs and larval stages in sheep. The larva, known as a *coenurus*, resembles a cysti-

cercus but has a number of scolices attached to a single bladder. In the brains of sheep the larvae produce a fatal disease. Occasional cases have been reported in man.

Taenia pisiformis is a common tapeworm of dogs and cats, the larvae developing in the abdominal cavity of rabbits. T. taeniaeformis occurs commonly in the intestine of cats. The larvae, strobilocerci, normally develop in the liver of rats or mice. There is considerable evidence that in the rat the larvae produce malignant tumors. These have also been produced by larval extracts in the absence of the living parasite and once developed are metastasizing and transplantable.³⁶ This species is of interest for a number of immunological phenomena. Definite sex resistance is shown by the fact that female rats acquire fewer larvae from a given dose of eggs than do males. Castration of females or injection of male sex hormones reduces their natural resistance, while female hormones increase the resistance of the males.²³ Acquired immunity is dependent in part on antibodies transferable with serum. Two antibodies are detectable. One acting early in the infection prevents development of the larvae; it is elicited by both infection and vaccination and is removed from the serum by absorption with worm substance. The other acts late in the infection, aborting the development of the larvae in the liver; it is elicited by infection but not by artificial immunization and is not absorbable with parasite material.

Dipylidium caninum, a common parasite of the intestines of dogs and cats, measures 10 to 50 cm. in length. Each elongate, rounded mature proglottid contains two sets of reproductive systems, one opening on each side of the segment. The uterus breaks up into pouches, each containing several eggs. Dog lice and various species of fleas serve as intermediate hosts, the cysticercoids in the insects being infective when ingested. Young children occasionally harbor the adult tapeworm. 106

Diphyllobothrium latum. The fish tapeworm Diphyllobothrium latum (or Dibothriocephalus latus) is the most important human parasite in the order Pseudophyllidea. It differs markedly in structure and life cycle from the species discussed above. It is a more primitive type, in many respects showing strong resemblances to the trematodes.

Morphology and life cycle. The adult worm in the intestine of man is very large,

often exceeding 10 meters in length. The scolex is an elongate ovoid structure, about 1 by 2.5 mm. in dimensions, bearing, instead of the circular suckers of the cyclophyllidea, two elongated grooves which serve as attachment organs. The mature proglottid is broader than long, about 4 by 10 mm. in size. The small testes are scattered throughout the lateral dorsal regions, emptying by fine ducts into the vas deferens. This coiled tube runs from the posterior center of the segment forward to the muscular cirrus. which opens into the ventral genital pore in the middle of the anterior region. The vagina extends from the genital pore back to the large oötype in the center of the posterior region. The ovaries lie on either side of the ootype, and the vitellaria are small follicles scattered throughout the lateral ventral regions. Arising from the oötype, the eggfilled uterus coils throughout the center of the proglottid, ending at the uterine pore just behind the genital pore. This permits escape of the eggs without rupture of the proglottid, so that, contrary to the usual situation as seen in T. solium, the eggs are released into the intestinal contents and escape in the feces. The spent proglottids at the end of the worm break off and are passed in the feces.

The egg is undeveloped when it escapes in the feces, embryonation requiring a week or more in water. Like the eggs of most trematodes it is ovoid, with an operculum. Average measurements are 65 by 45 μ . Hatching occurs by opening of the operculum to release a free-swimming, spherical, onchosphere covered with long cilia. This embryo, the *coracidium*, dies within a few days un-

less it is ingested by a suitable small crustacean, one of several species of copepods of the genera Cyclops and Diaptomus. Losing its cilia, the embryo reaches the body cavity of the copepod and becomes, in two to three weeks, an elongated solid body with a round tail bearing the six hooks seen in the embryo. This stage, the *procercoid*, is about 0.5 mm. long.

If the infected copepod is eaten by a fish, the procercoid penetrates the wall of the intestine and reaches the viscera or muscles. Here, in a week or more, it transforms into the *sparganum*, a worm-like stage 0.5 to 2 cm. long with a rudimentary scolex at the anterior end. This stage, ingested by man in undercooked fish, develops to the adult worm in the intestine, and eggs may be found in the feces after three weeks or more. If the infected fish is eaten by another fish, the spargana become established in the tissues of the second fish and remain infective for man. Human infections have been known to persist for years.

The human disease. D. latum commonly produces multiple infections, a number of worms occurring in the same intestine. The adult worms usually cause no symptoms, though in heavy infections intestinal disturbances may occur, sometimes with generalized edema. A small proportion of infected individuals show severe anemia, indistinguishable from pernicious anemia. It has been shown that the tapeworm concentrates vitamin B₁₂ in its tissues, and it is clear that the consequent deprivation of a susceptible individual precipitates the anemia. Satisfactory data on acquired immunity are not available. Laboratory

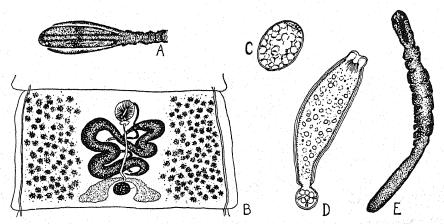


Figure 244. Diphyllobothrium latum. A, scolex, \times 10. B, mature proglottid, \times 6. C, egg, \times 250. D, procercoid, \times 100. E, sparganum, \times 8.

diagnosis depends on identification of the eggs in fecal smears. As with the other tapeworms, quinacrine hydrochloride is the drug of choice.

Epidemiology and control. The adult worm parasitizes man, cats, dogs, bears, and other fish-eating mammals, but man is probably the principal source of infection in endemic areas. Susceptible copepods are widespread, and a variety of fishes can serve as second intermediate hosts. Endemic centers are known in northern and central Europe, northern Asia, Japan, parts of Africa, and the Great Lakes region of North America. Infection is especially widespread in Finland, where the incidence has been estimated at 20 to 25 per cent and where most of the cases of tapeworm anemia have been reported. Personal protection depends on thorough cooking or freezing of fish from endemic areas and care in handling of such fish. Other control methods are aimed at reducing the pollution of bodies of water with untreated human feces and at the supervision of transportation of possibly infected fish.

Related species. Several related species of tapeworms, normally parasites of lower animals, are reported occasionally in man. Man also occasionally harbors the spargana of several species of Diphyllobothrium, normally parasites of the dog and other carnivores. D. mansoni is a common example. 107 Infection with these larvae in the subcutaneous tissues, muscles, or eye is

known as sparganosis. Among the larvae infecting man is the very rare parasite, Sparganum proliferum, which multiplies enormously in the subcutaneous tissues and may cause death. The adult of this species is unknown. Larval infections with Diphyllobothrium usually result from ingestion of copepods in drinking water. In parts of the Orient split frogs are sometimes used as poultices, and human sparganosis has resulted from the migration of spargana from the frog tissues into the exposed tissues of man.

NEMATODA

All the important human parasites in the phylum Nemathelminthes (Aschelminthes of some authors) belong to the class Nematoda. They are cylindrical, elongated worms, unsegmented and with a body cavity which is not lined with a peritoneum of mesodermal origin as in higher animals. Symmetry is primitively bilateral. Male and female reproductive systems usually occur in separate individuals noticeably different in general appearance.25, 133 In some parasitic forms, parthenogenesis and protandry occur. Free-living nematodes are among the most numerous of animals and are found in a tremendous variety of habitat. Parasitic species in plants and animals are also abundant, and wild vertebrates are usually parasitized as are many invertebrates. Nematodes

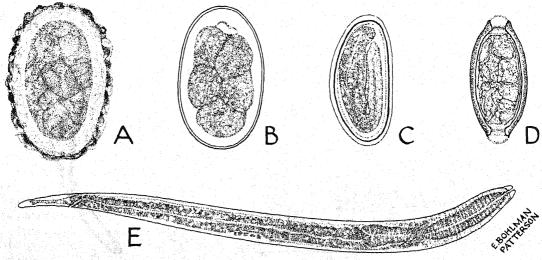


Figure 245. Nematode eggs and larvae. A, Ascaris lumbricoides. B, Necator americanus. C, Enterobius vermicularis. D, Trichuris trichiura. E, Strongyloides stercoralis (rhabditiform larva). × 400.

are important as disease agents of most domestic animals and the majority of mankind is infected with one or more species.

The structure of the nematodes parasitizing man varies greatly, and in size alone species occur which are too small to be readily seen with the unaided eye as well as forms over one meter in length. The life cycles are also varied from simple direct forms of development to those requiring several hosts. Among the simple forms, the pinworm of man, *Enterobius vermicularis*, will serve to illustrate the morphology and life history.

A word of caution is necessary here. The order of presentation of species in this section, based primarily on life cycles, cuts sharply across the lines of zoological classification, separating closely related types and bringing together types of very different structure.

Enterobius vermicularis. The human pinworm, Enterobius vermicularis, normally inhabits the upper large intestine of man, occasionally invading the female genitourinary system. The female worm, about 1 cm. long by 0.5 mm. in diameter, is a spindleshaped organism with a long, pointed tail (see figure). The outer covering is a smooth, impervious, flexible cuticle, characteristic of nematodes in general. This cuticle, together with the fact that the body musculature is exclusively longitudinal, gives the worms a characteristic bending, wriggling type of movement. Among nematodes the cuticle shows great variety of external structures - knobs, fins, papillae, etc. - which are of value in technical identification of species. The alimentary tract consists of a mouth at the anterior end, a muscular esophagus with a posterior bulbous enlargement, and a thin-walled intestine emptying through the anus near the posterior end. The female reproductive system empties through a short vagina a little in front of the middle of the body. Extending forward and back from the vagina are two separate eggproducing systems (one to several in other nematodes). Each consists of a broad uterus filled with eggs, followed by a short narrow oviduct, which terminates in a long, thin ovary coiled about in the body cavity.

The male worm is smaller than the female, 2 to 5 mm. in length and 0.1 to 0.2 mm. in diameter, the posterior end coiled into a tight spiral and lacking the pin-like tail of the female. The male reproductive system is a single tube consisting of a testis, a vas deferens, a seminal vesicle, and a muscular ejaculatory duct, provided with glands, which empties with the intestine into a cloaca. A sharp copulatory spicule can be extruded through the anus. The posterior end has minute expansions (more prominent in many other nematodes) which form a clasping bursa used in copulation.

Some of the eggs escape in the intestine, passing out in the feces, but usually the gravid females migrate out of the anus to release their eggs on the surrounding skin. The eggs average 55 by 25 μ in size, usually show mature embryos when passed, and are infective within a few hours. Ingested by man they hatch in the small intestine, where the worms mature and mate before passing down to the large intestine. Eggs appear in the feces about two weeks after infection. The adults in the intestine are short-lived, but infection is maintained or increased in the individual by self-infection. Hatching of eggs on the perianal surfaces with subsequent migration of larvae into the anus (described as "retroinfection") occurs in related species and it has been suggested this may also occur in man.

Pinworm infection in man. The adult worms in the intestine have little or no effect on the host. However, they occur in the

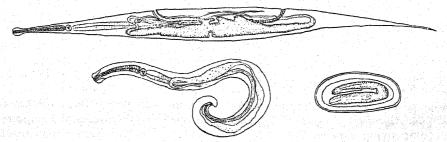


Figure 246. Enterobius vermicularis. Top, female, × 15. Left, adult male, × 15. Right, egg, × 300. (Redrawn from Faust after Leuckart.)

vermiform appendix, and since they are said to be more common in diseased than in normal appendices, they have been suspected of an etiological role in appendicitis. During the nocturnal emergence of the female and with egg deposition in the perianal region, itching occurs which may be intense, leading to scratching, scarification, weeping eczema, and bacterial infection. Worms may enter the female genitalia and cause similar irritation and occasionally migrate into the peritoneal cavity via the fallopian tubes. In children pruritus ani, sleeplessness, and resulting irritability are common. A slight eosinophilia may accompany infection and no immunity is known to develop.

Diagnosis is based on identification of eggs in the feces or from the perianal skin. The best method utilizes a strip of cellophane tape. The gummed side is pressed repeatedly on the perianal skin and then applied to a microscope slide for examination. Piperazine and pyrivinium chloride are therapeutically effective, but treatment is often followed by reinfection from the environment.

Epidemiology and control. Man is the only host of E. vermicularis. The eggs are resistant to drying and may contaminate clothing, bedding, and house dust, thus maintaining a constant source of infection in homes and institutions. The parasite is cosmopolitan. It is especially common in children, probably largely because their insanitary habits allow greater chances of spread. Treatment of cases is useful in control, provided sanitary measures are taken to minimize reinfection from the local environment.

Related species. Similar nematodes occur in many lower animals and are sometimes economically important as minor disease agents. Syphacia obvelata, a species found in rats and mice, is an occasional parasite of man and may readily be obtained for study from rodents. The eggs resemble those of E. vermicularis but are twice as large.

Trichuris trichiura. Trichuris trichiura is a frequent inhabitant of the cecum, appendix, and upper colon of man. The basic morphology of T. trichiura is similar to that of Trichinella spiralis, which is illustrated in an accompanying figure. The anterior three-fifths of the adult worm, containing he nonmuscular esophagus, is attenuated,

and the general appearance of the body has given rise to the common name "whipworm." Male and female worms are similar in size, 3 to 5 cm. long. The adult worms, embedded in the intestinal mucosa, have been accused of a role in the pathogenesis of appendicitis, but the evidence is inconclusive. Light infections are common throughout the population of the world's tropics and subtropics, are relatively without symptoms, and are unimportant to the infected individual. However, heavy infections, particularly in children, cause a hyperemic mucosa of the colon and rectum, chronic diarrhea, extensive inflammation, and rectal prolapse.⁸¹ Consistently effective drugs were not available until very recently, when it was shown that dithiazinine, a cyanine dye, is highly efficient in eliminating the worms from the intestine. The eggs passed in the feces are thick-walled, with a plug at either end, 53 by 22 μ in size. They are very resistant, surviving for months in contaminated soil. The embryos, undeveloped at the time of passage, require two weeks to several months, depending on temperature and humidity, to reach the infective stage. Ingested with contaminated food or water, they produce adult worms in the intestine. T. trichiura infection is world-wide but especially abundant in the tropics and subtropics because of poor sanitation and the effect of climate on development of the eggs.

Ascaris lumbricoides. Widely known simply as the "roundworm" of man, Ascaris lumbricoides is a large parasite of the small intestine. The white or flesh-colored females measure 20 cm. or more in length by 5 mm. in diameter, the males, 16 cm. by 3 mm. The characteristic eggs, 45 to 75 μ by 35 to 50 μ , have a smooth inner and a roughly tuberculated outer shell. They are undeveloped when passed in the feces. In soil or water they become infective in nine days or more, depending on environmental conditions. They may survive for several years despite drying, bacterial contamination, or adverse chemical conditions. Ingested in food or water they hatch in the intestine. The embryos, however, do not develop directly into adults in this site. Gaining access to the circulation they are carried to the lungs and escape into the air spaces. Via the trachea they reach the pharynx and are swallowed. Partial development occurs during the above migration. It is

completed in the small intestine, where egg-producing adults are found about two and one-half months after infection.

Passage of the migrating larvae through the lung may produce severe bronchopneumonia. Larvae filtered out of the circulation in abnormal sites induce inflammatory lesions. Adult worms in the intestine often cause no symptoms. Particularly in heavy infections, however, intestinal and nervous disturbances may occur, and intestinal obstruction occasionally results. Massive infection of children in the tropics is common with numerous fatalities prior to the availability of piperazine. The drug acts as a myoneural blocking agent for Ascaris and the paralyzed worms are eliminated by normal peristalsis as they are no longer able to maintain their position in the intestine. Consequently, chemotherapeutic methods now are effective in infections which previously necessitated surgical intervention to eliminate intestinal obstruction by worms. Various other injuries result from migration of the adult worms out of the normal habitat into the appendix, the bile ducts, the upper alimentary or respiratory tracts, the genitourinary system, or through the intestinal wall into the abdominal cavity. Hypersensitivity develops in infected persons and in laboratory workers who have handled the worms, and severe anaphylactic reactions may occur in such individuals.

Acquired immunity has not been observed in man, but experimental animals exhibit partial resistance to reinfection with related parasites. Antibodies are detectable by various tests after either infection or vaccination with worm extracts, and considerable investigation has been devoted to antigenic analysis. An allergic skin test has been used in diagnosis but is often positive in uninfected individuals who have acquired hypersensitivity from contact with the parasite. Laboratory diagnosis is preferably based on the finding of eggs in stools. However, a small proportion of cases harbor only male worms, and in such cases the immunological tests can be used.

Piperazine and hexylresorcinol are effective in removing the adult worms from the intestine. Some other drugs, particularly those used for treatment of hookworm infection, may cause migration of the adult Ascaris into abnormal sites.

Man is the only known host of A. lumbricoides. Infection predominates in children; that this results in part from age resistance is suggested by the marked age resistance of lower animals to related parasites. Infection is world-wide but most prevalent in the tropics and subtropics, where sanitary and climatic conditions favor its dissemination. Control depends on the reduction of soil contamination by proper sewage disposal and treatment of infected individuals. Raw vegetables are an important source of infection and should be thoroughly washed before consumption.

Related species. Cats, dogs, horses, and other lower animals harbor a number of closely related species. Rare human infections have been reported with at least three of these. A distinct race of A. lumbricoides causes severe pneumonitis in young pigs, known as "thumps." In man the eggs hatch and the larvae reach the lung, but adult worms do not become established in the intestine. Similarly, the human A. lumbricoides develops only to the lung stage in pigs. The adults of the pig Ascaris are easily obtained from slaughterhouses for laboratory study.

Visceral larva migrans. Visceral larva migrans has been recognized only recently as a clinical entity and the nematodes responsible are close relatives of Ascaris. The species most often involved are Toxocara canis and T. cati, the extremely common ascarids of the dog and cat. The larvae do not mature in man but wander extensively

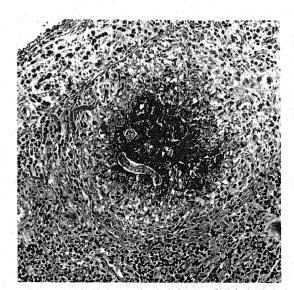


Figure 247. Human visceral larva migrans. Liver section showing granulomatous area containing *Toxocara canis* larva. × 105. (Beaver.)

through the tissues producing lesions and granulomata in the brain, eye, liver, lung, or other sites. The symptoms produced may be minor except for eosinophilia or may involve hepatomegaly, pulmonary infiltration, cough, hyperglobulinemia, and allergic manifestations.⁷⁷ Diagnosis is difficult but may be made on recovery of larvae in sectioned biopsy material with recognition of the larvae as shown in the accompanying figure. Serologic reactions are of value in diagnosis but give cross-reactions with Ascaris.85, 132 There is no specific therapy. Children with a history of dirt eating and an association with household pets run a risk of serious infection, emphasizing the need for anthelminthic treatment of dogs and cats.

Large nematode larvae causing eosinophilic granulomas and acute abdominal syndromes in man have been identified as Anisakis. This relative of the ascarid worms is generally considered to be a parasite of marine mammals, and in areas of Europe and the Far East where raw marine fish are eaten, this type of visceral larva migrans is being reported. 155, 170

Hookworms. The two important hookworms of man, Ancylostoma duodenale and Necator americanus, will be discussed together. Known since antiquity, human hookworms were first accurately described by

Dubini in 1843. Their relationship to disease was definitely shown by Perroncito in 1880, and the life cycle was elaborated by Looss.

Characteristics and life cycle. The adults of A. duodenale from the human small intestine are shown in the accompanying figure. The female measures 1 to 1.5 cm. in length by about 0.5 mm. in diameter. The double reproductive system coils abundantly throughout the body cavity. Two pairs of prominent unicellular glands are seen in the anterior half of the body, one excretory in function, the other secreting histolytic enzymes. The dorsally directed mouth is characterized by two pairs of ventral teeth (replaced in N. americanus by curved cutting plates). The male measures a little less than 1 cm. in length by about 0.4 mm. in diameter. The reproductive system shows a long, muscular ejaculatory duct, a prominent seminal vesicle, and a coiled testis. At the posterior end of the male worm is a broad, flat clasping apparatus, the copulatory bursa, composed of cuticle supported by finger-like rays of tissue. A. duodenale and N. americanus can be identified by details of structure of this bursa as well as by the differences in buccal teeth noted above.

The thin-walled eggs measure about 38 by 58 μ . Those of the two species of human hookworms cannot be distinguished micro-

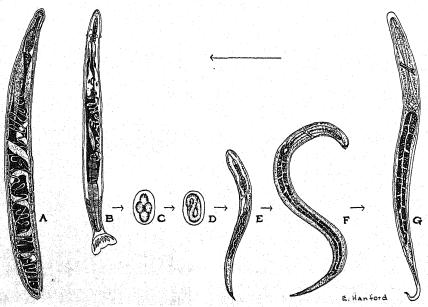


Figure 248. Life cycle of a hookworm, Ancylostoma duodenale. Note differences in magnification. A, female worm. B, male worm. C and D, eggs. E, first stage larva. F, second stage larva. G, infective larva. A and $B \times 10$. C to $G \times 250$. (Adapted from Looss.)

scopically from one another. They are usually in early stages of development upon escape from the body, maturing and hatching within 24 hours if conditions are favorable in the soil. The larva, about 0.25 mm. in length, feeds on fecal material and grows in three days or more to a length of about 0.4 mm. After molting it continues to grow until. by the fifth day or later, it measures about 0.6 mm. These two stages, characterized by a bulbous esophagus, are known as rhabditoid larvae. The third stage, resulting from a second molt, is a thinner form with a closed mouth and a less bulbous esophagus. It is usually still surrounded by a "sheath," the molted skin of the preceding stage. This is the filariform larva, the infective stage for man. It does not feed but may survive long periods in the soil under favorable conditions. If the infective larva comes in contact with human skin, it actively penetrates through the epidermis, reaches the circulation, and is carried to the lungs. Here, like the migrating larva of Ascaris, it breaks out into the air spaces and reaches the intestine via the trachea and esophagus. After two further molts, the worms are mature. Egg production begins about one month after infection.

Hookworm disease. Penetration of the skin by hookworm larvae produces a dermatitis known as ground itch or miner's itch. This is severe enough in some previously exposed individuals to be incapacitating. The adult hookworms live attached to the mucosa of the small intestine, feeding on blood and bits of tissue. They move about frequently, leaving bleeding wounds in the intestinal mucosa. The chronic loss of blood causes anemia and edema, resulting, in severe cases, in retardation of growth and mental development and in weakness and general debilitation.48 In many areas where hookworm disease is of importance, this anemia is intensified by virtue of the fact that nutritionally the population receives a relatively inadequate supply of available food iron. The additional loss of large quantities of blood and iron through hookworm infection produces the severe anemias often seen.125 Intestinal disturbances are seen in heavy infections. Death, except in infants and young children, is rarely a direct result of hookworm disease, but the condition often contributes to death from other causes. In white persons the symptoms are directly related to the intensity of infection, which,

because of the necessary development outside the body, is stable unless re-exposure occurs. Individuals with fewer than 25 N. americanus virtually never show symptoms. Those with between 25 and 100 worms show borderline effects. With more than 100 worms some clinical injury is almost always detectable. A. duodenale is more harmful, about half as many worms being required to produce a given effect. Normally, infection is maintained by constant re-exposure. In previously infected persons living under conditions which prevent additional infection, however, it has been shown that about one-half of the worms are lost in six months and about three-quarters in two years. A smaller number persist for as much as 12 years.

Immunity. Puppies can be largely protected against the dog hookworm by repeated small infections. Similar partial protection has been reported in a human volunteer. That antibodies are involved is shown by the fact that some protection is conferred on puppies by immune serum.

Diagnosis. Diagnosis hookworm of infection is based on recovery of characteristic eggs from the feces. Direct fecal smears were formerly used, but many infections with fewer than 25 worms are not detected with this method. Because the number of hookworms present in an infected individual is so important, several quantitative diagnostic methods have been devised. In the Stoll method a measured quantity (4 ml.) of feces is mixed with dilute NaOH solution (56 ml.), and all the eggs in a known sample volume (0.075 ml.) of this suspension are counted. The Lane method, or D.C.F. (direct centrifugal flotation), can be applied to lighter infections. One milliliter of feces washed by centrifugation is mixed with brine and recentrifuged with a coverslip on top of the tube. The eggs rise and can be counted on the coverslip. A relatively simple and accurate densitometric technique has also been devised.7 Figures obtained by either of these methods are expressed as the number of eggs per milliliter of feces. A single female N. americanus produces about 45 eggs per ml. of feces (about 6000 eggs per day) so that 25 worms (male and female) produce about 600 eggs per ml. and 100 worms produce about 2500 eggs per ml. A. duodenale produces about twice as many eggs per worm, but because of the greater pathogenicity of this species the egg output gives a roughly equivalent index of the severity of infection in both species.

A number of drugs are effective in eliminating hookworms from the intestine. The best at present are tetrachlorethylene and hexylresorcinol.

Epidemiology and control. Worms morphologically similar to N. americanus occur in pigs, but infection experiments indicate that they are distinct from the human hookworms. Man is the reservoir of infection with both A. duodenale and N. americanus.

There has been much study of the habits and requirements of hookworm larvae in the soil. The optimal temperatures are about 75° F. for A. duodenale and about 80° F. for N. americanus. Consistent high temperatures, about 100° F., are unfavorable. At 60° F. development is prolonged to two weeks or more, and below 50° F. there is little or no development. Continued temperatures below 40° F. kill the larvae. Moisture is essential to survival of the soil stages, but they die under water and are scattered by heavy rain. The type of soil is significant, probably largely in relation to its waterholding properties. Coarse sand and heavy clay are unfavorable, optimal development occurring in light sand or sandy loam. A large proportion of the infective larvae die within the first two weeks, but a few survive for several months under favorable conditions. Although lateral migration from the site of development is insignificant, the filariform larvae can move considerable distances vertically in the soil. Unless excessive drying occurs, they remain at the surface, where the chance of contact with human skin is greatest.

In addition to the environmental requirements of the larvae, various human factors affect the distribution of hookworm infection. Fecal disposal practices and the use of shoes are among the most important. All these factors combine to make hookworm infection a problem of the rural parts of warm countries. Exceptions occur in mines and tunnel-building operations, where local conditions may be favorable despite a generally unsuitable environment. N. americanus is the hookworm of central and southern Africa, the Western Hemisphere, and southern India. Throughout the rest of southern Asia it is mixed with A. duodenale. and a few foci of A. duodenale occur in South America. In most of Asia, southern

Europe, European mines, and North Africa only A. duodenale is found.

In the southern United States hookworm infection is most common in children, largely because adults are protected from infection by wearing shoes. That age resistance, if it exists at all, is a minor factor is shown by the fact that in countries where the whole population goes without shoes infection may predominate in adults. Racial immunity is marked, and Negroes under comparable conditions have fewer and less intense infections than do whites. Furthermore, Negroes seldom show clinical symptoms except in the presence of heavy infections.

Control depends on the reduction of soil contamination by treatment of cases and proper fecal disposal and on the prevention of contact between human skin and the soil. Chemical destruction of larvae in the soil has been of use only in particular cases. such as mines. Education is the major weapon in hookworm control, for the chief problems are sociological, and biological knowledge of the parasite is adequate to insure successful hookworm eradication if the habits of infected populations can be sufficiently influenced. Treatment of cases has an important place in hookworm campaigns. Formerly it was customary to treat all infected members of a community, or even the whole community (mass treatment), if sample diagnoses showed abundant and heavy infections. Current opinion favors treatment of only the clinically significant cases. In most regions the treatment of a small proportion of the population can eliminate most of the egg production.

Related nematodes of lower animals. Various animals harbor hookworms. Two species, Ancylostoma braziliense and A. caninum of dogs and cats, occasionally invade the skin of man. They are unable to develop further, and their wandering in the subcutaneous tissues produces a serpiginous dermatitis known as creeping eruption or cutaneous larva migrans. The adults of A. braziliense have been reported from man in the Far East. It has been claimed that the human cases harbor a distinct species, A. ceylonicum. Other, more distantly related parasites such as the numerous species of Trichostrongylus are disease agents of ruminants and are incidental parasites of man. One species, T. orientalis, is more common in man than in other animals. Heavy infections in man may produce diarrhea and high eosinophilia. The eggs resemble those of the hookworms but may be distinguished from them by their larger size and slightly different shape. The rat lungworm, Angiostrongylus cantonensis, has been found in human brain, where it causes a parasitic eosinophilic meningoencephalitis.³ In Asia and the Pacific islands this parasite is transmitted by the ingestion of raw land snails and slugs which are the intermediate hosts. It has been suggested that this organism is the cause of the "epidemic" eosinophilic meningitis of Tahiti.¹²⁶

Nippostronglyus brasiliensis (muris) of rats and mice is of interest because of extensive studies in the laboratory. Serum antibodies have been shown to exert a direct action on the parasites in vitro, and these antibodies together with tissue cells immobilize and destroy the larvae in the skin and lungs and destroy adults in the intestine.

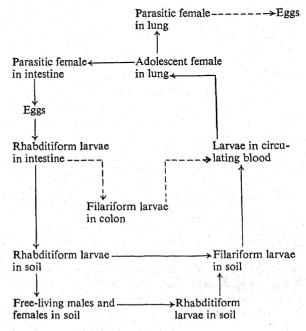
The first true cultivation of a parasitic worm has recently been accomplished with N. brasiliensis and Nematospiroides dubius. 164 Partial development of Necator americanus has been obtained. In a complex mixture of chick embryo extract, vitamins, amino acids, minerals, etc., the whole development of N. brasiliensis from the egg to the adult stage has been observed. While normal male and female worms were produced, the eggs produced were infertile. It is to be hoped that exploitation of this line of research will provide much needed information about parasitic worms.

Figure 249. Schematic life cycle of *Strongyloides stercoralis*. Broken arrows indicate rare or incompletely understood possibilities of development.

Studies have been made of the mechanisms involved in the exsheathment or cuticle shedding necessary between developmental stages of nematodes. The fluids responsible for this are believed important in stimulating the antibodies producing functional immunity to several of these infections. Extensive studies on the development of living nematode vaccines for use in veterinary medication are in progress, as in some forms it is believed that only antigenic stimulation by such metabolic products can produce significant immunity. 40, 142

Strongyloides stercoralis. Strongyloides stercoralis is a common intestinal parasite of man in warm countries. It exhibits a life cycle of extreme complexity, involving reproduction by adults in the soil as well as in the human intestine. Investigators working with different species of Strongyloides disagree on fundamental points, and it is not clear whether or not these disagreements result from specific differences in the worms studied.

Only female worms are known in the intestine of man. They are thin, transparent worms 2 to 2.5 mm. long by about 0.05 mm. in diameter. They lie buried in the mucosa of the small intestine, and the thin-shelled eggs are deposited in the tissues, where they hatch to release rhabditiform larvae very similar to those of hookworms. Escaping in the feces the larvae undergo one of two types of development. They may molt to filariform larvae which penetrate the skin



to establish infection. Alternatively, they may develop into the free-living males and females. The free-living female is stouter than the parasitic female and about 1 mm. long. The male is somewhat smaller, about 0.7 mm, in length, Eggs produced by the free-living female hatch to release rhabditiform larvae which may continue the freeliving cycle or may develop into infective larvae. The infective larvae, whether produced by free-living adults or developed directly from the rhabditiform larvae in the feces, penetrate the skin of man and reach the intestine via the circulation and lungs as in the hookworms. Male worms have not been found in the intestine, but "parasitic" males like those in the soil have been reported in the lung in abnormal hosts. Since investigation of a related species in rats has shown that a single infective larva can give rise to a female in the intestine producing fertile eggs, it is clear that the parasitic males are not essential in all species of Strongyloides. Oviposition in the lung has also been reported. A final complication is introduced by the reports that rhabditiform larvae might transform into infective larvae in the colon and penetrate its wall or the perianal skin to produce hyperinfections. The various paths of development indicated above are shown in the accompanying diagram. the dotted lines indicating phenomena which are incompletely known.

Penetration of the skin by the larvae causes dermatitis, and passage through the lung may give rise to bronchopneumonia. The parasitic females cause intestinal disturbances characterized chiefly by diarrhea. Laboratory diagnosis is based on identification of the rhabditiform larvae in the feces. These can be distinguished by the shorter buccal cavity from the rhabditoid larvae of the hookworms, which are occasionally encountered in fecal specimens. Dithiazanine is the most effective drug for therapy of S. stercoralis although not completely efficient in eliminating infection.

The epidemiology of S. stercoralis infection is similar to that of hookworm, and the same sanitary control measures apply. Dogs are infected with a strain indistinguishable from the form found in man and may be of some consequence in its maintenance. S. ratti of rats is a similar form which can produce local allergic manifestations in man and cutaneous eruptions similar to those of the hookworms.

Trichinella spiralis. The "trichina" worm. Trichinella spiralis, was first seen in human muscles at necropsy by Tiedemann in 1821. Leuckart and others worked out the life cycle.60 The adult forms are intestinal parasites of man and many other mammals, but their sojourn in the intestine is so brief that the more persistent larval infection in the muscles receives the main emphasis. The infective stage for man is a larva in the muscles of the hog. This stage is a minute coiled worm, about 1 mm, in length, enclosed in a lemon-shaped fibrous cyst, 0.25 by 0.5 mm. in dimensions. Ingested by a susceptible host the cysts reach the stomach, where the muscle and cvst wall are digested away. In about two days the larvae mature in the small intestine to adult males and females. The male is a thin, transparent worm about 1.5 by 0.04 mm. (Fig. 250). The anterior half of the body is occupied by a nonmuscular esophagus like that of the closely related Trichuris trichiura. At the posterior end are two pear-shaped clasping lobes. The female is about 4 by 0.06 mm, in size. After fertilization the females burrow into the mucosa where, after about one week, they begin to deposit minute larvae, about 0.1 mm. in length, in the tissues. These larvae reach the general circulation and are filtered out in the skeletal muscles, cardiac muscle, the central nervous system, and other sites. In most tissues they die, but in skeletal muscle they grow in about two weeks to the infective stage. One month after infection the larvae are surrounded by a fully developed cvst wall, produced by the host tissues. Much later, probably only after death of the larvae, these cysts become calcified, but they are known to remain infective at least for several months. Any skeletal muscle may be infected, but the larvae are most abundant in the diaphragm, intercostals, tongue, larynx, and eye muscles.

Meanwhile the adults have disappeared from the intestine. Most of the males die and are passed out within a week after infection. The females have largely disappeared within a month. It will be seen that the complete life cycle occurs in a single individual, for infective larvae are produced in the tissues of the same host which harbors the adults in its intestine.

an pron man infection in a population may be high, the usual infection with Trichinella is without detectable symptoms and the damage to the

host is probably insignificant. However, severe cases resulting from relatively massive infection occur and are characterized by two phases. Intense infection with the adult worms produces gastrointestinal disturbances, usually with diarrhea. Occasionally the injury to the intestine is so severe as to result in death within a few days after infection. Muscle invasion by the larvae leads to varied signs of toxemia, hypersensitivity, and muscle injury. Heavy infections cause death or chronic illness. The severity of the disease is proportional to the number of larvae ingested, and many mild cases undoubtedly escape detection.

Immunity to superinfection is demonstrable in experimental animals and can be transferred with immune serum. Some protection may be produced in the laboratory by vaccination with killed larvae, but this is insufficient to be of practical value. Direct action of immune serum on the worms has been described. Precipitin and skin tests are available using extracts of larvae digested out of infected muscle. These reactions are detectable two to three weeks after infection and remain positive for several years. Slide flocculation tests with antigen coated on bentonite particles or on cholesterol are the preferred immunological reactions for diagnosis. Like the complementfixation test they become negative a few months after infection and are thus indicative of current or recent trichinosis. The immunological and serological aspects of trichinosis have been the subject of a recent review which summarizes the numerous techniques which have been employed in diagnosis.83 Eosinophilia, often over 20 per cent, is characteristic and offers presumptive indication of trichinosis. Other diagnostic methods involve search for the larvae in body fluids or tissues. During migration they may be found occasionally in blood or spinal fluid, but it is often impossible to detect them. Muscle biopsies show the larvae in severe cases. There is no specific treatment for trichinosis. Supportive treatment and the use of ACTH ameliorates to a large extent the toxic febrile stage of this disease. In addition, there has been some use of thiabendazole, which suggests that it may be useful in treatment of this disease in the acute state.143

T. spiralis occurs principally in man, hogs, rats, and bears, although experimental infection has been produced in many species of mammals and birds. Human infection follows the consumption of undercooked pork (more rarely the meat of other animals, such as bears), while pigs acquire the parasite from garbage containing pork scraps or,

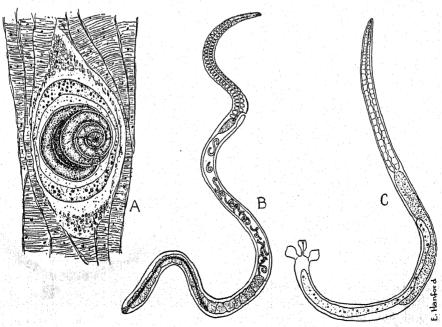


Figure 250. Trichinella spiralis. A, larva in muscle, \times 100. B, adult female, \times 50. C, adult male, \times 50.

less commonly, by eating carcasses of infected rats. Infection through the ingestion of larvae which pass out in feces has also been demonstrated and may be of some importance in the infection of coprophagous swine. In the United States as well as in the artic regions a significant sylvatic infection in carnivores exists. T. spiralis is worldwide in distribution, though infection in the tropics is rare. Various surveys in the United States, using routine necropsy examination of human diaphragms, have revealed a general infection rate of between 14 and 28 per cent despite the fact that very few clinical cases are diagnosed. 60 More recent studies indicate that the true incidence at present may be much less than this, more nearly approaching one-third of this figure.

Personal protection can be assured by thorough cooking of pork products, including hamburger of unknown composition. Government meat inspection does not attempt to detect Trichinella, for examination guaranteeing safety is considered impractical, but refrigeration for 24 hours at -18° C. destroys most of the larvae. Laws which forbid the feeding of raw garbage to hogs have been successful in reducing the incidence of infection. New methods of food preservation with gamma radiation show promise for control of trichinosis. Treatment of meat with radioactive cobalt renders the worms infertile, so that they cannot produce larvae. This prevents not only the systemic effects but also the intestinal pathology of early trichinosis.

THE FILARIA

Seven species of nematodes belonging to this group are important parasites of man. All produce motile embryos termed microfilariae which are produced in large numbers by the female in the host tissues and are released within a sheath (believed to be an elongate eggshell) or unsheathed. Microfilariae migrate or are carried by the circulation to the skin or to peripheral vascular beds, where they may be ingested by bloodsucking arthropods. Within this insect intermediate host the microfilariae transform without multiplication into infective larvae. become associated with the insect mouthparts, and may then be transmitted by the insect to the definitive host. After an incubation period and growth, which is little understood and may take as much as a year. the filaria may be found in their final sites of parasitism and are producing larvae. Wuchereria bancrofti and Brugia malayi adults parasitize lymphatic vessels; Onchocerca volvulus is usually in subcutaneous nodules or in deeper connective tissue as is Acanthocheilonema streptocerca. Mansonella ozzardi and Acanthocheilonema perstans are found in pleural or peritoneal cavities, while Loa loa as an adult migrates extensively through the connective tissues.

wuchereria bancrofti. Of the several species of filaria mentioned above, Wuchereria bancrofti is by far the most important. The disease, elephantiasis, was known in antiquity, but the larvae were first seen in 1863 by Demarquay and the adults by Bancroft in 1876. Manson, in 1878, showed that development of the larvae took place in mosquitoes. This discovery is noteworthy because it represents the first incrimination of an insect in the transmission of disease.

Characteristics and life cycle. The adults live coiled in the lymph nodes of man. They are thread-like translucent worms, the female 7 to 10 cm. long by 0.25 mm. in diameter, the male 4 cm. by 0.1 mm. The egg, covered by a thin membrane, hatches in the uterus or in the host tissues to release an active embryo about 0.2 mm. long. This embryo, known as a microfilaria, escapes into the lymph and enters the circulating blood by way of the thoracic duct. In most regions the embryos of W. bancrofti show

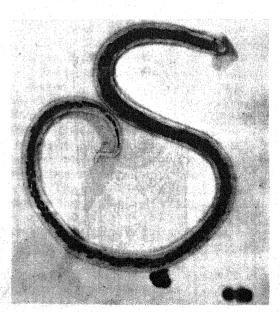


Figure 251, Microfilaria of Wuchereria bancrofti in thick blood film. × 300.

marked nocturnal periodicity, occurring in the peripheral blood almost exclusively at night. In many of the South Pacific islands, however, they appear in the blood at all hours. Extensive study has failed to provide a satisfactory explanation of this periodicity, although there is suggestive evidence that daytime activity induces accumulation of the microfilariae in capillaries of the lung. 63

The microfilaria develops no further in the circulating blood. If ingested by a susceptible mosquito, however, it undergoes a series of transformations in the tissues of the insect. Within a day the larva leaves the stomach of the mosquito and invades the thoracic muscles. Here it develops into a stout "sausage larva," which, after a second molt, elongates to form the infective stage, a thin worm 1.5 to 2 mm. in length. This infective larva leaves the muscles and migrates to the proboscis sheath of the mosquito, arriving six days or more after the infective blood meal. When the mosquito takes a new blood meal, the infective larva, apparently stimulated by the warmth of the skin, breaks out of the proboscis sheath and penetrates the skin, probably through the wound caused by the biting mosquito, to establish infection in man. The early development of the worms in man is not known, but microfilariae appear in the circulating blood several months after infection. In the related species, Brugia malayi (see below), development to the adult stage requires about 80 days in experimental animals. Although the longevity of the adult worms in the lymphatics has not been determined, they probably live for several vears.

Filariasis. The adult worms are most prevalent in the lymph nodes of the inguinal region, but they are also found in lymphatics elsewhere in the body, they induce inflammation and fibrosis in the infected nodes. The consequent restriction of lymph flow causes edema, lymphangitis, lymphadenitis, and elephantiasis. Secondary infection of the lymphatics with streptococci and staphylococci is often significant. Long-standing cases sometimes show enormous enlargement of the scrotum, vulva, legs, or breasts.

Evidence of acquired immunity is lacking, but complement-fixing antibodies are detectable in the serum, and skin tests are positive in infected individuals. The reactions are group-specific, occurring with extracts of filarial worms from lower animals and giving false positives in other

nematode infections. Laboratory diagnosis is preferably based on the identification of microfilariae in fresh blood preparations or stained thick blood films. They are often not detectable in early infections or in long-standing cases with much tissue damage. Other filarial worms infecting man (see below) also produce microfilariae in the circulating blood, but these can be differentiated microscopically from the larvae of *W. bancrofti*.

Certain antimony compounds, notably Neostibosan and Anthiomaline, eliminate microfilariae from the blood of cases for long periods. Hetrazan, an oral drug, is more effective and less toxic and has been widely used, principally because of its ease of administration and its ability to minimize mosquito infections by eliminating microfilariae in the human reservoir. It is not known whether these drugs kill the adult worms in man, but they destroy the adults of other filarial worms in experimental animals.

Epidemiology and control. Man is the only known host of the adult worm. A large number of mosquitoes of the genera Culex, Aedes, Mansonia, and Anopheles support development of the larvae, but the most important are Aedes polynesiensis in the South Pacific islands, Anopheles gambiae in West Africa, and Culex quinquefasciatus (C. fatigans) elsewhere. There is an interesting correlation between the habits of the mosquito hosts and the periodicity of the microfilariae discussed above. Aedes polynesiensis bites by day, and it is in the regions where this mosquito transmits W. bancrofti that the microfilariae are found in the peripheral blood at all hours. In other regions, where the principal vectors attack. man in the evening or at night, the microfilariae show a marked nocturnal periodicity.

W. bancrofti is a parasite of warm countries, occurring widely throughout the tropics and subtropics. Climate favors the production of mosquitoes and the development of larvae of W. bancrofti in the insect, and the poorer housing of the tropics permits greater contact between mosquitoes and man. The slow development of human infections, the lack of multiplication of W. bancrofti in the mosquito hosts, and other hazards combine to limit dissemination of the parasite. As a consequence the infection seems to die out in an area unless there are many human cases and abundant mosquitoes. The former endemic center in the United States, around Charleston, South Carolina, where the incidence was 20 to 30 per cent before 1920, now shows no cases. Control is a matter of mosquito reduction, protection from mosquito bites, attention being directed to the habits of the particular vectors in any area, and chemotherapy of cases.

Other species of filarial worms. Brugia malayi is very similar to W. bancrofti and is widely distributed in southeast Asia, the China Sea area, and Eastern India, often occurring with W. bancrofti. In some areas it is transmitted by mosquitoes of the genus Mansonia whose larvae obtain oxygen from subaquatic vegetation, posing additional problems of control. A similar parasite, B. pahangi, of Malaya is common in wild and domestic mammals and, in man, may produce the symptoms of tropical eosinophilia.

Onchocerca volbulus is widely distributed through central Africa, and in the Western Hemisphere has been introduced into Guatemala, Mexico, Venezuela, and Surinam. 120 The adults are found in tightly coiled masses in subcutaneous nodules of connective tissue where commonly they may be extirpated by minor surgery. Others may be deep-seated and not palpable. The microfilariae are not found in the circulating blood but concentrate in the skin, where they may readily be demonstrated by superficial biopsy. In the Western Hemisphere nodules containing the adults are found on the head, and the microfilaria cause ocular lesions commonly leading to blindness. Sensitization reactions are usual in infection with Onchocerca and may be pronounced following chemotherapy with Hetrazan.

Blackflies of the genus Simulium, whose larvae must develop in fast-flowing mountain streams, serve as intermediate hosts. Since man is the only known definitive host, attempts have been made to provide control by surgical removal of the onchocercal nodule plus chemotherapy. Intravenous Suramin (Naphuride sodium) is effective.

Loa loa is found in the subcutaneous tissues of a variety of primates including man and is limited to West and Central Africa, where it is transmitted by the biting deerfly Chrysops. It has been called the "eye worm" because of the frequency with which it may be found passing under the conjunctiva of the eye surface, and it is also associated with a fugitive "calabar" swelling presumed to be due to reaction of the host to the metabolic products of the migrating

adult worm. The microfilariae are diurnal, in the peripheral circulation, and unsheathed.

Acanthocheilonema streptocerca is found in the dermal and subcutaneous connective tissue and its unsheathed microfilariae are in the superficial layers of the dermis. It is limited in distribution to parts of Central Africa, A. perstans has a wide distribution in tropical Africa and is also present in coastal areas of South America and the West Indies. Its microfilariae are nonperiodic and unsheathed, and are found in the peripheral blood. The adults are found associated with the peritoneal cavity, in mesentery, in retroperitoneal tissues, in the pericardium, or occasionally in subcutaneous cysts. Infections are often without symptoms but may be associated with sensitization phenomena and rarely with more serious or fatal complications. Mansonella ozzardi is also found the tropical areas of the Western Hemisphere, occupies body cavities, and has circulating, unsheathed, nonperiodic microfilariae. No symptoms are associated with infection. Both species Acanthocheilonema and Mansonella of are transmitted by gnats of the genus Culicoides.

There are numerous filarial worms of mammals and birds widely distributed over the earth. One of these, Dirofilaria immitis, is an important pathogen of dogs, the adult worms living in and occluding the vessels of the heart. Antigen prepared from Dirofilaria has been used in intradermal and serological tests for the diagnosis of filariasis in man. The specificity of the reactions is low. Litomosoides carinii, a parasite of the body cavity of the cotton rat, is a form commonly used in laboratory investigations.

Dracunculus medinensis, the Guinea worm, is a common parasite of man in the Middle East, Africa, India, and Indonesia. It was also endemic in the tropics of the Western Hemisphere but has not been recently reported. Although a tissue parasite resembling the filaria, it does not have a true microfilarial stage. The small male worms are rarely seen. The females, after several months' development in internal connective tissues, appear in the subcutaneous tissues, usually of the leg. They are large worms, averaging about 1 meter in length, often visible through the skin as they lie in the tissues. The skin of the host ulcerates at the anterior end of the worm, and larvae escape, usually when the leg is subtuffer to 1854 the letter of

merged in water. These larvae develop in copepods, e.g., cyclops, and human infection follows ingestion of the parasitized copepods in drinking water. The human infection, possibly the "fiery serpent" of the Bible, is associated with severe sensitivity reactions immediately preceding initial blister formation and larval release by the female. In some regions the female worm is gradually removed from the tissues by rolling it up on a stick. Local injection of phenothiazine is said to be effective in therapy, and antihistamines may be used to relieve the sensitivity phenomena.

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Chapter Thirty-five

RICKETTSIA

The name rickettsia is applied to a group of very small, gram-negative coccobacillary microorganisms which includes the etiological agents of the typhus fevers, spotted fever and related diseases, and tsutsugamushi disease of the Far East.5, 54, 70 They were first observed by Ricketts in 1909 associated with Rocky Mountain spotted fever. Those found in the bodies of lice taken from typhus fever patients by da Rocha Lima in 1916 were named by him Rickettsia prowazeki in honor of Ricketts, who had died in 1910 of typhus fever during an investigation of that disease, and of von Prowazek, another early worker in the field who died of typhus. Closely similar microorganisms are found as the cause of heartwater disease in cattle and of other diseases of lower animals, including sheep and dogs, and also, as apparently nonpathogenic forms, in the sheep ked, Melophagus ovinus and from Haemaphysalis and Dermacentor ticks.9

Relation to bacteria and viruses. rickettsiae are of special interest, not only as the causative agents of human disease, but because they appear to represent transitional forms between the bacteria on the one hand, and the viruses on the other. As described elsewhere (Chap. Nine), the bacteria may be arranged in a series, from the purely saprophytic nonpathogenic forms through those which are saprophytic but pathogenic because they form potent toxins, to the closely adapted parasites which make up the majority of the disease-producing bacteria. Many of the last have fastidious nutritive requirements, reflecting limitations in synthetic abilities, and certain forms, especially Pasteurella tularensis and Bartonella, grow within the host cells, but all of these, with exceptions such as the leprosy bacillus and the spirochetes of syphilis and related diseases, are cultivable on lifeless mediums.

The rickettsiae, like the viruses, are set apart from the bacteria by their inability to grow in the absence of the living host cell, necessitating culture in the embryonated egg or in some other form of tissue culture. While it is a reasonable inference that they are dependent upon the host cell for essential metabolic reactions rather than essential metabolites, the rickettsiae do show limited metabolic activity and resemble the bacteria in having a similar cell structure and multiplying by a process of binary fission. They are related to the viruses through the organisms of the psittacosis group for which there is evidence for the occurrence of fission during replication, but which contain only fragments of enzyme systems, viz., cytochrome C and folic acid. It is perhaps relevant that infections with rickettsiae and the psittacosis group are susceptible to treatment with the antimicrobial chemotherapeutic drugs, while the diseases of viral etiology are not.

Although fragments of enzyme systems, such as flavin and biotin, are associated with certain of the poxviruses, and adenosine triphosphatase activity is apparently an integral part of one of the avian leucosis viruses, most other viruses show little or no evidence of independent metabolic activity. Also, there is no evidence that any of these replicate by a process similar to fission. Rather, the viral particle seems to develop by differentiation within a morphologically discrete portion, or matrix, of the protoplasm of the host cell as in the case of the poxvirus and related viruses, or final assembly seems to occur just prior to ex-

826 RICKETTSIA

trusion of the complete viral particle from the host cell as in the replication of influenza virus (Chap. Four).

The rickettsiae, then, although occupying an intermediate position, seem to be more nearly related to the bacteria than to the viruses, but whether such a graded series of microorganisms has phylogenetic implications is another matter (Chap. Seven).

Morphology. These microorganisms may appear either as cocci or short bacilli, 0.3 to 0.5 μ long and 0.3 μ in breadth, but bacillary forms as long as 2 μ may occur, and filamentous forms have been described. The cells occur singly, in pairs perhaps to be associated with binary fission, and often in dense irregular masses, within affected cells, particularly those of mesothelial origin which line the serous cavities. Some species are found only in the cytoplasm, but others, such as those of the spotted fever group, occur in the nucleus as well. They are nonmotile and do not show obvious capsules.

In electron micrographs of isolated rickettsiae prepared in the usual way there appears to be a dense central portion surrounded by a peripheral area of less dense material, but when the preparation is fixed with osmic acid, or is a section of infected tissue, the dense central portion is not differentiated. A well-defined cell wall is present and demonstrable in electron micrographs. Staining. The rickettsiae stain poorly or not at all with the usual bacterial stains but are readily stained with Giemsa, are stained red against a blue background with Macchiavello's stain (basic fuchsin, citric acid, and methylene blue) and a light blue against a pink background with Castaneda's stain (buffered methylene blue and safranine counterstain). They may also be stained with Wright's stain in tissue sections to give a blue nucleus and pink cytoplasm. The coccoid and coccobacillary forms stain uniformly, but bipolar staining of the bacillary forms is not uncommon.

Chemical composition. The chemical composition of typhus and Q fever rickettsiae is closely similar to that of most bacteria. There is some uncertainty as to the precise amounts of the various components present because of possible, or even probable, loss during purification procedures such as, for example, ether extraction and washing. Preparations not treated with ether have given about 30 per cent protein. 47 per cent lipid including 30 per cent neutral fat and 17 per cent phospholipid, and 12 per cent nucleic acid. The lipid figure is in contrast with that of 12 to 13 per cent found in ether-extracted and washed preparations, but it is uncertain as to whether the differential represents contamination or rickettsial lipid removed by the extraction. The nucleic acid fraction contains both the deoxypentose and pentose types, in a ratio of

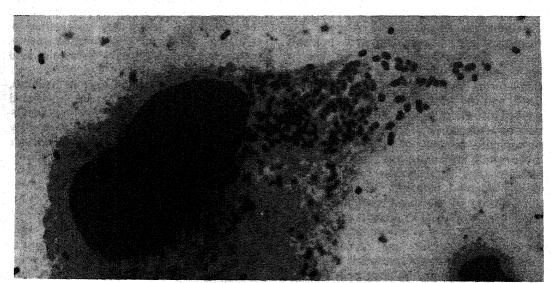


Figure 252. The rickettsiae of tsutsugamushi in scrapings from a mesothelial surface of an infected guinea pig. Note the coccobacillary forms, often paired, and their intracellular location. \times 2000.

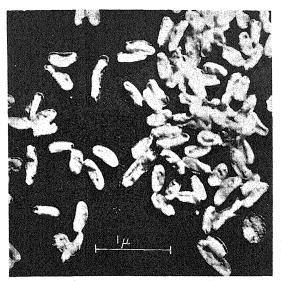


Figure 253. Electron micrograph of Q fever rickettsiae purified by elution from a cellulose anion exchanger. (Hoyer *et al.*²⁶)

about 2:1, with relatively constant amounts of DNA, but variable amounts of RNA from one preparation to another. Cell wall preparations from rickettsiae lysed with deoxycholate resemble bacterial cell walls in composition, containing amino acids and a polysaccharide moiety hydrolyzing to glucose, galactose, hexosamine, and glucuronic acid. The close similarity to bacterial cell walls is also indicated by the demonstration of muramic acid, probably in association with mucopeptide, in rickettsial cell walls. 52

Physiology.¹⁵ The occurrence of independent metabolic reactions of rickettsiae has been noted above. Largely through the work of Boyarnick, Wisseman, and others, it has become clear that these microorganisms carry out glutamic-aspartic acid transamination and oxidize glutamic acid via the citric acid cycle. It has been found also that glutamate oxidation can lead to the phosphorylation of hexose and the formation of adenosine triphosphate from the diphosphate. The formation of glucose-6-phosphate is catalyzed by a hexokinase which is similar to hexokinase from other sources. 50 A terminal respiration mechanism is indicated by the presence of flavin-iron-porphyrin catalysts. The available evidence suggests that the Krebs cycle may be operative, and pyruvate the chief energy source.47 Folic acid is present and a possible function is suggested by the synthesis of serine from glycine and formaldehyde in the presence of tetrahydrofolic acid⁴⁶ by the Q fever rickettsia. There is evidence too that host-independent pyrimidine synthesis may occur.⁴¹

These activities are not stable and the conditions effecting their inactivation and reactivation are of considerable significance. On storage in isotonic salt solution in the cold, rickettsiae lose the ability to carry out the above reactions and at the same time lose toxic and hemolytic activity (see below) and infectivity. It has also been known from early work on spotted fever by Parker and others that the rickettsiae in starved ticks are avirulent but become virulent after the tick is fed or incubated at body temperature. Loss of toxic and hemolytic activity also occurs at 36° C. in the absence of metabolizable substrate.

In the first two instances, physiological activity, toxic and hemolytic activity, and infectivity are restored by incubation of the microorganisms with NAD and/or coenzyme A.^{11, 12} Para-aminobenzoic acid has a rickettsiostatic effect (see below), and this inhibitory effect is also reversed by NAD.²⁵ The inhibitory effect of PABA is thought to be due to the formation of a PABA-NAD complex, thus preventing dehydrogenase activity, and reactivation occurs when exogenous NAD is added to the system.

Inactivation of toxic and hemolytic activity at or near body temperature is possibly different. It is prevented in the presence of glutamate, pyruvate, or ATP, and reactivation occurs on incubation with glutamate and inorganic phosphate and is increased by ATP, the gain or loss of activity being correlated with ATP. Evidence such as the foregoing provides a rational basis for the nature of the suspending medium, a sucrosephosphate-glutamate solution, used for rickettsiae.

Cultivation. As indicated above, the rickettsiae grow only in the presence of, and probably invariably within, host cells. They will grow on the chorioallantoic membrane of the embryonated hen's egg, but usually produce only small numbers. As first shown by Cox, they grow readily and profusely in the yolk sac of the embryonated egg, and this method of culture has been one of the most widely used.

They also grow in various kinds of tissue culture. In modified Maitland medium, proliferation of the rickettsiae is not associated with active cell metabolism, as is

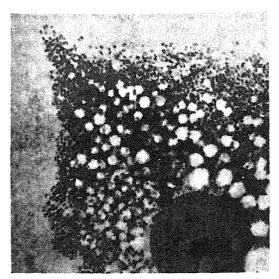


Figure 254. Rickettsia prowazeki in an infected cell from chick endoderm cultured in vitro. Most of the cytoplasm of the cell is occupied by rickettsiae. × 1500. (Weiss.)

usually the case with the viruses, but rather with a low metabolic rate of the host tissue, and proliferation is most rapid as the host cells begin to die. Active multiplication has, in fact, been observed in dead chick embryos which contain some surviving cells.

Several species of rickettsiae have been studied in cultures of mouse fibroblast tissue culture, using a cell line (MBIII) that grows in roller tubes as a dispersed suspension of individual cells.13 The rate of growth was relatively slow, with a generation time of about 16 hours, which compares with the short generation time of rapidly multiplying bacteria of 20 minutes or less, and the longer generation time of 30 hours or more of slow-growing bacteria such as tubercle bacilli. Studies of growth in rat fibroblast culture in the living state by phase microscopy have made possible the direct observation of the occurrence of binary fission.60 It was also observed that, following intracellular multiplication, the microorganisms are extruded from the host cells in association with a process of extension and retraction of microfibrils at the surface of the cell. It is probable, however, that rickettsiae are usually released by degeneration and dissolution of the host cell. Cultures of explants of the endodermal cells of the chick yolk sac have also been used for the cultivation of rickettsiae.72,76 and may be used for quantitative titration of infection, at least with the Q fever rickettsiae, because

foci of infected cells are readily observed in stained preparations and may be counted.

Resistance. The resistance of the rickettsiae to deleterious influences is generally of the same order as that of the more delicate bacterial vegetative cells. They are killed by the usual disinfectants but, like some of the viruses, are resistant to glycerol, and by heating at 55° to 60° C. for 30 to 60 minutes. The Q fever rickettsiae are said to be somewhat more resistant to heating, and these and the typhus fever rickettsiae are relatively resistant to drying, persisting in viable infectious form in the airborne state in the first instance, and in infected louse feces in the second, for considerable periods.

Pathogenicity. The rickettsiae appear to be well-established parasites of arthropods, and there is good evidence for the acarinid origin of the rickettsial diseases. They are not pathogenic for the insect vectors that transmit them, except lice, and, in fact, the infection is congenital in insects undergoing an incomplete metamorphosis, e.g., ticks. They also appear to be reasonably well adapted to certain animals, especially rodents, which in many instances constitute a reservoir of infection in nature.

The characteristic pathological changes found in rickettsial disease result from the multiplication of the microorganisms within the endothelial cells of the small blood vessels throughout the body, and especially in the skin and brain in the case of typhus fever. Proliferation of affected cells, and thrombosis following injury to the lining of small vessels, is common. Inflammatory cells accumulate around areas of vessels, and in severe infections in man and guinea pigs, e.g., those of spotted fever, the smooth muscle cells of the vessel media may be involved, and extensive thrombosis can lead to gangrene.

Toxin. Two kinds of rickettsial toxicity are demonstrable, the one an endotoxin associated with the intact microorganisms^{6, 16} and the other a hemolytic activity which lyses sheep and rabbit cells but not those of a number of other species examined. The endotoxin is lethal for mice and is titrated by intravenous inoculation of material such as yolk sac culture, with deaths up to 18 hours attributable to toxicity. The titer of the toxin is directly proportional to the number of rickettsiae present and is correlated with their viability. Most agents that destroy infectivity also inactivate the toxicity, with the exception of ultraviolet

radiation which, in appropriate doses, selectively reduces infectivity without affecting toxic, hemolytic, or respiratory activity.2 The activity of the toxin is similar to that of other microbial endotoxins and increases vascular permeability but appears to differ in other respects.78 It is specifically neutralized by antiserum, with cross-reactions between members of the main groups of rickettsiae, the typhus, spotted fever, and tsutsugamushi groups (see below), but not between groups. There is also an exotoxic activity demonstrable by intradermal inoculation of the rabbit to produce a local inflammatory reaction and by enlargement of the liver and spleen of rats and mice on intraperitoneal inoculation.

The hemolytic activity of suspensions of purified rickettsiae is species-specific, as noted above, and is closely associated with both endotoxin and enzymatic activity, particularly the oxidation of glutamate, and its activity is inhibited by metabolic inhibitors. Ordinarily hemolytic activity of the causative microorganism does not play an appreciable part in the pathogenesis of infectious disease, but is concerned in fatal rickettsial toxemia in the rabbit also, the animals showing massive intravascular hemolysis, with hemoglobinemia, hyperkalemia, myocardial damage, and hypotension. It is not clear, however, that either the toxicity or hemolytic activity plays any significant part in the pathogenesis of rickettsial infections, for they appear to be dissociable from virulence.55

Experimental infections. Rickettsial infection may be produced in the guinea pig, the usual procedure in isolation being the intraperitoneal inoculation of 5 ml. of blood into each of two animals. A febrile attack occurs in seven to 12 days, and some varieties of rickettsiae produce a characteristic orchitis or scrotal reaction which has differential value. An acute fibrinous exudate is formed in the scrotal sac, and many cells packed with rickettsiae may be found. The disease in the guinea pig is often not fatal, and this animal is much more resistant to infection than is man. Other common laboratory animals are more resistant, and rats, mice, rabbits, dogs, cats, etc., may show no febrile reaction or other outward manifestation of disease, though the infection has been established and in some cases, at least, may persist for months.

Chemotherapy. Sulfonamides and penicillin are ineffective in the chemotherapy of the rickettsial diseases, although they have been used in the past in the treatment of complications such as bronchopneumonia and other secondary bacterial infections. Para-aminobenzoic acid has been found to be partially effective in typhus and spotted fevers if given early, but cannot be regarded as a really effective chemotherapeutic agent. With the development of the broad-spectrum antibiotics effective chemotherapy of the rickettsial infections has been made possible, for these substances have been found to be effective chemotherapeutic agents for this group of diseases. Chloramphenicol has been of particular interest in the treatment of tsutsugamushi disease (scrub typhus), since effective prophylactic immunization of man is not yet practical and the mite reservoir of infection is not subject to adequate control. Largely through the work of Smadel and his co-workers63 it has been found that the clinical disease may be suppressed by prophylactic or therapeutic administration of this substance but that relapse occurs unless the treatment is continued for about four weeks; apparently the antibiotic alone does not suffice to eliminate rickettsiae from the tissues, but an immune response is required also. The tetracyclines have been found to be even more effective chemotherapeutic agents.

It has been of considerable interest that, although drug-resistant strains of rickettsiae have not yet been found in nature, Weiss and his colleagues have been able to develop resistant strains in the laboratory. Strains of *R. prowazeki* resistant to the rickettsiostatic activity of PABA, quinoxaline, and

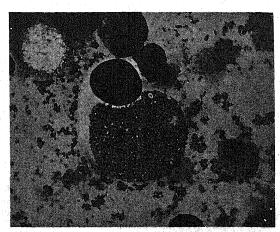


Figure 255. Rickettsiae of Q fever in a smear of skin exudate. Note the macrophage packed with enormous numbers of the rickettsiae. × 1125.

erythromycin have been produced by serial egg passage in the presence of increasing concentrations of the drug.^{73, 74, 75} Most, but not all, of the resistant strains did not appear to be altered in virulence and toxicity.⁷¹

Immunity. Recovery from an attack of rickettsial disease usually confers a solid and lasting immunity. In at least some instances this is associated with the prolonged survival of the microorganisms in the body as indicated by the occurrence of Brill's disease (see below) and the demonstration of tsutsugamushi rickettsiae in clinically recovered individuals.

The development of artificial immunization procedures will be considered later, but it may be noted here that the immune response is manifested by the appearance of protective antibody, of opsonins and agglutinins for the rickettsiae, and of complement-fixing antibody. The last has been of a good deal of interest as a differential diagnostic method and distinguished even closely related varieties of rickettsiae. Antibody neutralizing the toxicity of typhus rickettsiae occurs also and is used in the United States as the basis of assay of immunogenic potency of vaccines. The rickettsiae contain both somatic and capsular antigens. In the purification of yolk sac culture the material is extracted with ether to remove lipid material, and, with the exception of R. orientalis, the rickettsiae are found in the aqueous phase. The extraction results in the liberation of soluble, group-specific antigen in the aqueous phase which has been found to be stripped-off capsular material.

The Weil-Felix reaction. In some rick-

ettsial diseases agglutinins appear to certain strains of Proteus. This apparently anomalous response was observed by Weil and Felix in 1915 and has very considerable diagnostic value. They found that a strain of Proteus vulgaris, which they designated X-2, was agglutinated by typhus serums, and another strain, designated X-19, was similarly agglutinated but to much higher titer; in fact, titers as high as 1:50,000 are occasionally observed in European typhus. It was further shown that the agglutinating antigen was a part of the heat-stable somatic or O antigen; these strains are, therefore, commonly termed OX strains. Other strains showing this immunological characteristic have been isolated since. It is of some importance that an O antigen be used in carrying out the agglutination because antibody to the flagellar antigen of Proteus occurs with some frequency in normal serums. More general application of this agglutination test, commonly known as the Weil-Felix reaction, showed that it is specific for the typhus group of fevers, serums from other rickettsial diseases agglutinating only to low titer or not at all. The phenomenon is a consequence of the presence of a common antigen in the typhus rickettsiae and the X strains of Proteus.

The Proteus agglutinin is only a part of the antibody response. It is not identical with the rickettsial agglutinin for the latter appears earlier, persists longer, and appears to be associated with protective antibody. Furthermore, the opsonin remains in typhus serum after it has been absorbed with Proteus.

The SECREPT AND A COURSE OF THE PARTY OF THE

Immunological (Weil-Felix) Grouping of Rickettsial Diseases*

	IMMUNOLOGICAL GROUP	
OX-19 OX-19 ++++ OX-2 + OX-K —	OX-K OX-19 – OX-2 – OX-K ++++	Undetermined OX-19 + OX-2 + OX-K +
Classic, European typhus Brill's disease Endemic murine typhus of United States, Australia, Greece, Syria, Manchuria, Malay (shop ty- phus), India, Burma, Philip- pines, Hawaii, Toulon (fièvre nautique)	Tsutsugamushi of Japan, Formosa, Malay, Netherlands East Indies "Scrub typhus" or "rural typhus" of Malay, N.E.I., French Indo-China, India, Australia Mite fever of Sumatra	Spotted fever São Paulo typhus Fièvre boutonneuse Fièvre exanthematique Febbre errutiva Tick fever of South Africa Kenya fever Tick typhus of India

^{*}Modified from Felix.

Another immunological type of Proteus, the OX-K strains, was found to be agglutinated by tsutsugamushi serums but not by typhus serums. This, coupled with the OX-19 type, makes possible the division of the rickettsial diseases into three groups: the typhus group, in which OX-19 is agglutinated; the tsutsugamushi group, in which OX-K is agglutinated; and the spotted fever group, which is indeterminate in that neither type of Proteus is agglutinated to high titer. A classification of the rickettsial diseases on this basis is given in the accompanying table.

The significance of the Weil-Felix reaction is wholly unknown. The occurrence of one common antigen, or more specifically a polysaccharide haptene, in two such different microorganisms might be a matter of chance. The occurrence of a second common antigen, shared by another variety of Proteus and another group of rickettsiae. on the basis of chance alone is improbable. There is no evidence, however, which points to any other connection between Proteus and the rickettsiae. There is, for example, no association between the X strains and typhus; X strains are found in diseases other than typhus, and Proteus strains other than X strains are found in typhus. Proteus infections, of course, often result in the formation of specific agglutinins. It has been found, for example, that the serums of persons infected with Pr. vulgaris, Pr. mirabilis, or Pseudomonas aeruginosa will agglutinate OX-K strains of Proteus to a high titer. Such persons might be said to give a positive Weil-Felix reaction.

Specific serological reactions. An accurate method of serological diagnosis and identification should be based on the use of rickettsial antigen and thus a true immunological specificity. Although it has been extremely useful, the Weil-Felix reaction is not a specific reaction and is not completely reliable. The protection test in the guinea pig is useful in the demonstration of antigens functional in effective immunity, but the relatively greater natural resistance of these animals as compared with that of man tends to blur differentiation in some instances. There is, for example, a significant adequate cross-protection between louseborne and murine varieties of typhus rickettsiae in the guinea pig, but not in more highly susceptible man. Similarly, the protection test does not differentiate between strains of O fever rickettsiae.

The *in vitro* serological reactions, made practicable by the availability of relatively large amounts of antigen from yolk sac culture, are of two kinds, the one direct agglutination of rickettsiae in suspension, and the other complement fixation. Two kinds of antigen are present, group-specific soluble antigen and strain-specific antigen present in the microorganisms.

Yolk sac cultures contain large amounts of lipid material which is removed by ether extraction of a saline suspension of the yolk sac material. As indicated above, all the rickettsiae except the tsutsugamushi rickettsiae, which are found in the interphase emulsion, remain in the aqueous phase, and the treatment releases soluble antigen from all of these except Q fever rickettsiae. The soluble antigen can be separated by spinning out the rickettsiae in the centrifuge and used in a precipitin test, or the mixture of rickettsiae and soluble antigen can be used as an antigen in the complement-fixation test.

Difficulty has been experienced with some antigen preparations in that they tend to give false positive complement-fixation reactions. especially with Wassermann positive serums. This is not true of suspensions of washed rickettsiae, but these are difficult and laborious to prepare. Satisfactory soluble antigens may be prepared from infected yolk sacs purified by ether extraction, followed by treatment with benzene and precipitation with sodium sulfate. Complement-fixation with antigen does not give false positives with Wassermann positive serums, but does not always distinguish between epidemic and murine typhus.

The fluorescent antibody technique has been applied to the demonstration of rickettsiae by the direct method, 14, 33 and to the titration of antibody by the indirect method. Troublesome cross-reactions in the latter, as between murine and epidemic typhus, are largely eliminated by the simple expedient of diluting the serum to be tested in heterologous soluble antigen. 22

Classification. The intermediate position of the rickettsiae with respect to other microorganisms has been described above, but it is also apparent that differentiation occurs within the group. In general, the rickettsiae pathogenic for man fall into four groups. Three of these are well defined with respect to immunological character and pathogenicity but are heterogeneous with

Rickettsial Diseases of Man

COMPLEMENT- FIXATION	Group- or species- specific		Group- or species- specific		
WEIL-FELIX REACTION	0X-19: ++++ • 0X-2: + 0X-K: 0	OX-19: ++++ OX-2: + OX-K: 0	0X-19; + 0X-2; + 0X-K: 0	0X-19; + 0X-2; + 0X-K: 0	
EXPERIMENTAL INFECTIONS AND OBSERVATIONS	G. pig; fever only	G. pig. fever and scrotal swelling	G. pig. fever and severe scrotal reaction (necrosis)	G. pig. fever and scrotal swelling	
VERTEBRATE RESERVOIR	Man	Wild rats Field mice	Incomplete evidence indicates: rabbits, small rodents, dog, opossum (S. America)	Dog Rodents? (S. Africa)	
ARTHROPOD VECTORS	Human louse, Pediculus humanus	Fleas Xenopsylla cheopis X. astia Rat louse Polyplax spinulosa	Ticks Dermacentor andersoni D. variabilis D. variabilis Rhipicephalus sangulueus Haemophysalis Ieporis-palustris Several species of Ambhyomna Ixodes dentatus	Ticks Rhipicephalus sanguhans R. apendiculatus Haemaphysalis leachii	
GEOGRAPHIC DISTRIBUTION	Europe, Asia, S. America N. America, Europe, etc.	World-wide	North America Brazil Colombia	Mediterranean littoral India Bast Africa South Africa Central Asia	
DISEASE	Epidemic typhus (classic, European, louseborne typhus) Brill's disease	Murine typhus, (endemic typhus, fleaborne typhus)	Spotted fever (Rocky Mt. spotted fever) São Paulo typhus Tobia fever	Boutonneuse fever (Marseilles or Mediterranean fever) Indian tick typhus? South African tick- bite fever? Siberian tick typhus?	
SYNONYMS		Rickettsia mooseri R. muricola	Dermacentrox- enus rickettsi	Dermacentroxenus rickettsi R. rickettsi Vat. conori Dermacentroxenus sibericus?	
NAME OF RICKETTSIA	Rickettsia prowazeki	Rickettsia typhi	Rickettsia rickettsii	Rickettsia conori	
	Typhus group		Spotted fever group		

				- [
		Not yet adequately studied	Specific	Not available
0X-19: + 0X-2: + 0X-K: 0	OX-19: 0 or + OX-2: 0 OX-K: 0	0X-19: 0 0X-2: 0 0X-K: ++++	OX-19: 0 OX-2: 0 OX-K: 0	OX-19: 0 OX-2: 0 OX-K: 0
G. pig; fever and scrotal swelling	Mouse; ascites, splenomegaly, death	Mouse; ascites, splenomegaly, death	G. pig; fever only	None
Rat? Marsupials?	House mice	Apodemus agrarius and other wild rodents Birds?	Bandicoot Australia) Birds; Human infec- funs occur from direct or indirect con- tect with in- fected sheep, cattle, goats	3
Tick? Isodes holocyclus?	Mite Allodermanyssus sanguineus	Mites Trombicula akamushi T. deliensis T. pallida	Ticks Haemaphysalis humerosa kodes holocyclus Dermacentor andersoni americanum americanum (and isolations from	Human louse, Pediculus humanus
Australia	Urban localities in N.E. United States and in USSR	Japan, Korea, China, Philip- pines, S.E. Asia, India, Indonesia, N. Australia	World-wide	Recognized only during World Wars I and II on European fronts
North Queensland tick typhus	Rickettsialpox	Tsutsugamushi disease Japanese river fever Kedani fever Scrub typhus Rural typhus	Q fever	Trench fever (Wolhynian fever; five-day fever)
		Rickettsia orientalis R. nipponica R. akamushi	Rickettsia burnetti R. diaporica	Rickettsia pediculi R. wolhynica
Rickettsia australis	Rickettsia akari	Rickettsia tsutsu- gamushi	Coxiella burnetii	Rickettsia quintana
		Tsutsu- gamushi group		

834 RICKETTSIA

respect to insect vectors. These are the rickettsiae responsible for the typhus group of fevers, those of the spotted fever group, and the causative agents of tsutsugamushi disease. There is some confusion as regards the names of diseases caused by these forms because a number of the spotted fever group of diseases have been given names such as "tick typhus." The rickettsiae causing Q fever and trench fever are set apart from the other rickettsiae, but are unrelated to one another, and make up a miscellaneous group.

Within the typhus group, two species are distinguished, R. prowazeki and R. typhi, the causative agents of epidemic louseborne or European typhus, and of murine typhus respectively. Four species are distinguished within the spotted fever group: R. rickettsii, the causative agent of spotted fever, São Paulo typhus, and Tobia fever; R. conori, the causative agent of Boutonneuse fever and the probable causal agent of tickborne diseases occurring in Africa, India, and Siberia under the general name of tick typhus; R. australis, the causative agent of Australian tick typhus; and R. akari, the causative agent of rickettsialpox. The tsutsugamushi rickettsiae are immunologically heterogeneous, but subgroups or species have not been distinguished, and these agents are given the single name R. tsutsugamushi.

The apparent causative agent of trench fever is poorly known, and the designation *R. quintana* is not based upon a detailed differential characterization. The causative agent of Q fever has, however, been characterized with some precision and is set apart from the other rickettsiae sufficiently that it has been given a different name, *Coxiella burnetii*. The occurrence and differential characteristics of these rickettsiae are summarized in the accompanying table.

The rickettsiae found in lower animals are also thought to differ from the human pathogens sufficiently to warrant different names: Cowdria ruminatum for the causative agent of heartwater disease, and others causing disease of lower animals are designated Ehrlichia, including Ehrlichia ovina found in sheep, E. canis found in dogs, and E. bovis found in cattle. The rickettsia-like nonpathogenic microorganism is found in the sheep tick and, unlike the rickettsiae cultivable on glucose-blood agar, has been named Wolbachia melophagi.

The Typhus Fevers 34

The typhus fevers constitute a group of closely related infections which occur in various parts of the world. The incubation period is five to 18 days, and the clinical picture is essentially the same in all the typhus fevers, though the severity of the disease varies widely. There is an initial violent headache which persists with the onset of chills and higher fever. A macular eruption appears soon after the fourth day which remains until defervescence. Crisis occurs at about the twelfth day, and recovery may be more or less complete at the end of another two weeks, though the cough which develops may persist, and mental vigor may remain somewhat impaired for some time. The complications include typhus gangrene, which is perhaps associated with the circulatory disturbances arising during the disease, a highly fatal bronchopneumonia, otitis media, and typhus encephalitis. Death rarely occurs before the end of the first week. The case fatality is

highly variable; it has been as great as 70 per cent in some epidemics, and 20 to 30 per cent is not uncommon, while in endemic typhus it is much lower, perhaps 5 per cent. Typhus is almost always a considerably milder disease in children than in adults.

Two species of rickettsiae are responsible for these diseases, and characteristically occur in the cytoplasm but not in the nucleus of invaded cells. They are associated with two epidemiologically different types of typhus fever. One is the classic European or epidemic typhus and the etiological agent is R. prowazeki (R. prowazeki var. prowazeki). The other is murine typhus, sometimes called endemic typhus, and the causative rickettsiae is R. typhi (R. prowazeki var. mooseri, R. muricola, R. mooseri). Both are immunologically homogeneous, and strains from all parts of the world appear to be substantially identical. These rickettsiae may be distinguished from one another by complement fixation and contain both common and individual antigens.

EPIDEMIC LOUSEBORNE TYPHUS FEVER

The classic form of typhus fever known for many years is the louseborne typhus of Central Europe, which persists in endemic foci in Russia and Poland and has occasionally broken out in major epidemic form from time to time during periods of stress. The disease is associated with overcrowding and filth and has been termed "camp fever" and "jail fever." Epidemics are not infrequent in both civil and military populations during time of war and may become very extensive; it is estimated, for example, that 315,000 persons died of typhus in Serbia in 1915 and that 25 million cases occurred in Russia in 1917 to 1921.

The disease is transmitted by the human body louse, Pediculus vestimenti. The head louse may also transmit the infection, but its importance in the spread of the disease is not established. During the febrile stage of the disease, rickettsiae are present in the blood, and the louse is infected by ingestion of blood. In the louse the rickettsiae infect the cells lining the gut, multiply, and are discharged in the feces after rupture of the infected cells, the louse becoming infective in six to eight days at 32° C. The salivary glands are not infected, congenital transmission of the infection does not occur, and the louse is eventually killed by the infection.

Most human infections are probably acquired by contamination of the louse bite with infected feces, but infection via the mucous membranes is possible. The rickett-siae persist in viable form in dried material such as louse feces, and accidental laboratory infections have occurred following inhalation of infectious materials.

Although bedbugs and ticks have been experimentally infected, it is probable that the louse is the sole vector of the disease under natural conditions.

Brill's disease. Louseborne European typhus of a mild type is endemic, though in late years decreasing in prevalence, in cities along the Atlantic Coast in this country. Long regarded by many as different from European typhus, Brill's disease has been shown to be louseborne and identical

with European typhus. In this country it has presumably been imported from endemic areas in Europe. It has also been found in Australia⁶⁹ in immigrants from Central Europe. Brill's disease, or latent typhus, has been observed in Europe, both in England³¹ and on the continent. Such latent typhus, or healthy carrier state, capable of infecting lice fed upon the infected individual, may provide the starting point of epidemics. Russian workers tend to discount such a source of infection, considering that apparent recurrence is a consequence of reinfection rather than relapse.⁵¹ Since the infection is not congenitally transmitted in the louse, and infected lice usually die within two weeks, it seems probable that the reservoir of infection occurs in man.⁵⁶ Such a reservoir of subclinical infection in Egypt, for example, is indicated by serological surveys.1 The reservoir known to exist in Central Europe may consist in part of latent infections, but the disease smolders in endemic form in relatively large numbers of mild infections. For example, an overall incidence of 24.5 per cent of positive Weil-Felix reactions was reported in healthy Russians with no history of typhus in the German-occupied area of Russia in World War II, rising from 11 per cent in the less than five-year-old group to 34.5 in the sixto 10-year-old group.

MURINE TYPHUS

The type of typhus fever which prevails in the southern United States and in Mexico, where it is known as *Tabardillo*, was long assumed to be a mild form of louseborne European typhus. The disease is associated with rats, and it has since become apparent through the investigations of a number of workers that the rat is a reservoir of infection in both this country and Mexico and that the disease is transmitted from rat to rat and from rat to man by the rat flea, *Xenopsylla cheopis*, and by the rat louse, *Polyplax spinulosa*.

The disease may also be transmitted by the human louse and, when a case occurs by transmission from the rat in a community where there are lice in abundance, it has been suggested that murine typhus may become an epidmic louseborne infection. Such epidemics occur from time to time in Mexico. Murine typhus occurs with considerable

836 RICKETTSIA

frequency in this country; approximately 20,000 cases were officially recorded from 1932 to 1941, and 5191 cases with 214 deaths were reported in 1945 from 44 states but predominantly from the southeastern states. Prior to 1946 it was generally believed that murine typhus was an under-reported disease in this country. Since that year it has been apparently declining rapidly, with 685 cases reported in 1950, 46 cases in 1961, and 28 in 1965. With attention directed toward the disease, fallacious diagnosis appears to have become more common, largely as a result of misinterpretation of low-titer Weil-Felix reactions. With the advent of specific complement fixation, many reported cases are not confirmed by this method. In a study in the southeastern states it was shown, for example, that 25 per cent of reported cases were definitely not typhus.58 A degree of control of the disease is possible by reduction of rat and rat flea populations, as indicated by a study in Georgia in which the incidence of human disease was reduced by 80 to 90 per cent.36

Murine typhus also occurs elsewhere in

the world and is known by various names. The urban type occurring in Malaya or "shop typhus," the mild form of the disease in the Mediterranean region known as Toulon typhus, and other infections such as Moscow typhus, Manchurian typhus, and the Red Fever of the Congo are all murine typhus identical or nearly so with the type found in the United States and Mexico.

Clinically murine typhus does not differ appreciably from the classic European type. It is sometimes said that the European disease is likely to be more often fatal, but this does not appear to be true; both forms are equally fatal in epidemic form and relatively mild in the endemic form.

Differences between R. prowazeki and R. typhi are demonstrable but not great. The murine rickettsiae produce a necrotic scrotal reaction in guinea pigs, while the European variety does not; the murine variety may be carried indefinitely in mice without alteration, but the European variety tends to degenerate; the rickettsial pneumonia and intraperitoneal multiplication in rats with the production of enormous numbers of

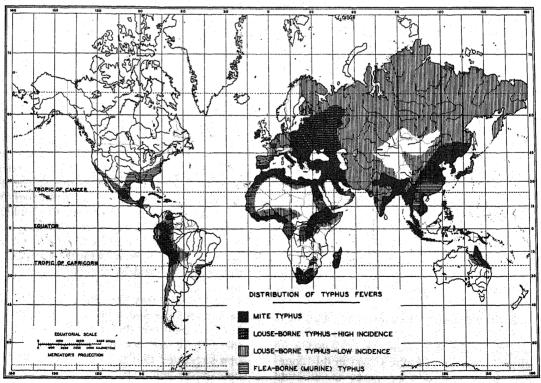


Figure 256. The world-wide distribution of typhus fevers, including classic or European typhus, murine typhus, and mite typhus of the Far East. (Redrawn from maps prepared by Army Medical Intelligence. Based on Goode Base Map No. 201M. By permission of the University of Chicago Press.)

rickettsiae may be produced by the murine variety but not by the European type alone. There is a slight immunological difference also, for, while recovery from either infection results in a solid and lasting immunity to both, murine vaccines protect against murine infection but only incompletely against infection with the rickettsiae of European typhus. The two may be differentiated by the complement-fixation test.

IMMUNIZATION

The preparation of vaccines which will produce an effective active immunity to typhus fever is a matter of some importance, since under the circumstances in which it occurs in epidemic form adequate control of the vector is often not possible. In man the immunity produced by immunization is specific, and the cross-immunity between epidemic and murine typhus is not of a sufficiently high order to be practical.

Inactivated vaccine. Rickettsiae have been grown in a number of ways for preparation of inactivated vaccines. Weigl vaccine was one of the first and was prepared from the bodies of lice infected by intrarectal inoculation. A vaccine prepared from the infected mouse brain has been used also. R. typhi produces a pneumonia in rats on intratracheal inoculation, and rickettsiae may be recovered in large numbers from infected lung tissue by differential centrifugation. The rat is not susceptible to this kind of infection with R. prowazeki unless its resistance is reduced by prior treatment such as x-irradiation. These methods were largely supplanted with the introduction of yolk sac culture for the preparation of vaccine in the early 1940's.

Of these the Weigl vaccine is impractical for use on any scale. Vaccine prepared from rat lung culture has been used to a considerable extent in Mexico and by the German army during World War II; the method of preparation of such vaccine, involving as it does intratracheal inoculation, is inherently highly dangerous and also relatively expensive.

The vaccine currently used in the United States is yolk sac vaccine, prepared by ether extraction as noted above, and the final preparation is a suspension of formalin-killed epidemic typhus rickettsiae and soluble antigen. There is some reason to believe that the

liberation of soluble antigen increases the immunogenic potency of the material. It is standardized on a toxicity basis, *i.e.*, prevention of the toxic effect of inoculated rickettsiae. It has been used in the United States Army, given as a course of three doses at seven- to 10-day intervals, followed by a single dose every four to six months. The possibility of allergic reactions following inoculation of the egg material has not proved to be important; approximately six to eight million troops received vaccine with only two fatalities attributable to it.

Experimental evidence strongly suggests that a marked degree of immunity is produced by immunization with this inactivated vaccine. There is no good field test of its efficacy, though only a very few cases of mild typhus and no deaths were recorded in the United States Army during World War II in spite of the exposure of many to the disease. There have been a number of instances of infection of vaccinated and unvaccinated laboratory personnel, and it is clearly apparent that immunization substantially modifies a subsequently acquired infection and the resulting disease is mild.

Vaccines prepared Attenuated vaccine. with attenuated microorganisms of sharply reduced virulence for man are a necessity in those instances in which the inactivated antigen will not produce an effective immunity, e.g., tuberculosis and yellow fever, or are desirable in those cases in which the induced immunity is of a low order as in plague. Multiplication of the attenuated microorganism provides a larger amount of antigen, undenatured by inactivation processes, and possibly additional antigenic substances formed during growth (Chap. Fifteen), but the possibility of reversion to virulence persists in the use of such vaccines.

In the case of R. prowazeki, a spontaneous change to avirulence for guinea pigs and other laboratory animals occurred during continuous egg passage of a strain isolated in Madrid and designated strain E. The possibility of this attenuated strain giving a superior effective immunity has been of considerable interest, and has been studied by Russian workers^{57,82} and by Fox and his associates in this country. 19, 20 In the latter studies human volunteers were used and subjected to extensive field trial in Peru, where the disease is endemic. Complementfixing and toxin-neutralizing antibodies are produced in more than 90 per cent of persons inoculated under field conditions. The im838 RICKETTSIA

mune response was shown to be associated with an effective immunity demonstrable by challenge inoculation with virulent *R. prowazeki* and to persist for more than six years

after immunization. There has been no indication of a reversion of the attenuated strain to virulence, nor do inoculated persons appear to serve as foci of infection.

The Spotted Fevers

The rickettsial diseases which make up the spotted fever group are considerably more heterogeneous than those of the typhus fever group. Several of them are but poorly understood as yet. All are transmitted by ticks, although some differ immunologically and clinically from Rocky Mountain spotted fever.³⁰

Rocky Mountain spotted fever. Clinically Rocky Mountain spotted fever resembles typhus fever; the rash is generally more extensive and the nervous symptoms may be more pronounced, but in areas where both diseases prevail it is exceedingly difficult to distinguish them on clinical grounds alone.

The disease has been known for many years in the Rocky Mountain region in states such as Idaho, Montana, Wyoming, Oregon, and Washington. In 1930, however, it was found in the eastern part of the United States in the South Atlantic states such as Maryland, Virginia, West Virginia, and North Carolina. Although the disease is not limited to these regions, of the 2190 cases reported from 1933 to 1937, 65.5 per cent were from the Mountain and Pacific states and 27.4 per cent from the South Atlantic group; the two areas combined accounted for 93 per cent of the total cases reported in the country. Approximately 200 to 300 cases are reported each year; 219 cases were reported in 1961, and 281 in 1965.

It is customary to speak of two types of the disease, an eastern type and a western type. Both are, however, immunologically identical and are caused by the same rickettsia, R. rickettsii, which, in contrast to the typhus rickettsiae, is found within the nucleus of invaded cells. Both are transmitted by ticks, but by different species. The wood tick, Dermacentor andersoni, is the vector of the disease in the western states, and the dog tick, Dermacentor variabilis, transmits the disease in the Atlantic region. The rabbit tick, Haemaphysalis leporis-palustris, disseminates the infection in the animal population. Amblyomma americanum, A. cajennense, D. occidentalis, Rhipicephalus sanguineus, D. parumapterus, and Ornithodorus parkeri are able to transmit the disease experimentally and are to be regarded as potential vectors. The infection is congenital, i.e., is transmitted from one generation to the next, in D. andersoni, but whether it is congenital in the dog tick is not clear. In the first instance, then, there need be no animal reservoir of infection although it is probable that animal reservoirs of infection occur.53,62 In the second, the dog may serve as a temporary reservoir of infection. The body louse may be infected experimentally with these and other rickettsiae of the group,77 but seems to play little or no part in the human disease.

The case fatality of spotted fever is highly variable. In Idaho it has been about 4 per cent, in western Montana about 20 per cent, and in certain regions of the Bitterroot Valley as high as 90 per cent. Over the country as a whole, however, the rate is 18 to 19 per cent. It is generally said that the disease is milder in the Atlantic region than in the Mountain states, but over a period of years there is little difference in case fatality; for the period of 1933 to 1937 the case fatality in the Mountain and Pacific states was 19.4 per cent and for the South Atlantic states 18.1 per cent.

Spotted fever was transmitted to guinea pigs and monkeys by Ricketts in 1907. Guinea pigs show a febrile reaction, the spleen is enlarged, and the necrotic scrotal reaction occurs. R. rickettsii is immunologically related to the typhus rickettsiae, but the titer of cross-reaction in the complement-fixation test is not high enough to cause confusion; there is also a small degree of cross-protection between the two.

Other spotted fevers. Closely similar tickborne disease has been observed in Central and South America; in Central America and Brazil it is known as São Paulo typhus, and in Colombia as Tobia fever. The causative rickettsiae are very closely related to, if not identical with, R. rickettsii. There seems to be no doubt that these diseases are spotted fever, and there is some tendency to refer to them collectively as American spotted fever.

Immunization. A solid immunity is associated with recovery from spotted fever, and an effective prophylactic immunity may be produced by inoculation with vaccine. The first vaccine was that prepared by Parker and his associates at the United States Public Health Service laboratory in Montana. It consisted of infected tick tissue inactivated with phenol and was used for more than 15 years. Subsequently it was displaced by formalin-inactivated vaccine of rickettsiae grown in yolk sac culture. These vaccines produce an effective immunity in experimental animals such as guinea pigs.

Vaccines are standardized for immunogenic potency in guinea pigs, *i.e.*, for active protection as measured by the average number of days of fever during the 12 days after challenge, and serum antitoxin 10 days after vaccination by passive protection of the mouse against toxin challenge. Active mouse protection against toxin challenge correlates well with the guinea pig test.⁸

While the incidence of the disease is too low to allow adequate field test, there is evidence that the naturally acquired disease in man is either prevented or modified by prophylactic immunization.

RELATED RICKETTSIAL INFECTIONS

There are a number of diseases of rickettsial etiology which are closely related to the American spotted fevers and which are known as tick typhus or tick fever and identified by the locality in which they are observed. The causative rickettsiae have been shown in some cases to be closely related to, but apparently not identical with, R. rickettsii, while others which are not this well known show clinical and epidemiological features which suggest a closely similar etiology.

Boutonneuse fever. This disease, also known as fièvre boutonneuse and Marseilles fever, is prevalent in the Mediterranean area, and is caused by *R. conori*. This rickettsia is immunologically related to, but separable from, *R. rickettsii* by toxin neutralization as well as by other serological methods. Cross-protection is practically complete in convalescent guinea pigs, but immunization with *R. conori* vaccine will not protect against *R. rickettsii* infection,

and immunological differences are also demonstrable by the complement-fixation test.

The animal reservoir of infection is the dog, and the disease is transmitted by the tick, *Rhipicephalus sanguineus*, in which the infection is congenital. The disease is milder than American spotted fever, with a case fatality rate of 1 to 2 per cent. It is distinguished clinically by the small, local, indurated lesion with a necrotic center which develops at the site of the tick bite,³² and is known as the eschar or tache noire, and by regional adenopathy.

Other tick fevers. The tick-bite fevers of Africa, including Kenya fever or Kenya typhus and South African tick fever, together with Indian tick typhus, appear to be substantially the same disease as boutonneuse fever, although there is no eschar in Kenya fever. In Africa the infection is found in a number of ticks, including *Haema*physalis leachii, Amblyomma haebreum, Rhipicephalus appendiculatus, and R. sanguineus, and the last is found infected in India also. Kenya fever is transmitted by R. sanguineus, but more often by the larvae of A. haebreum. While these vectors constitute a reservoir of infection, there is also evidence that a reservoir may be present in wild rodents.

North Queensland tick typhus. This disease, occurring in Australia, was first described in 1946. The causative rickettsia is immunologically related to the spotted fever rickettsiae but is sufficiently distinct that it has been designated *R. australis*. The evidence for tick transmission is as yet circumstantial but suggests that *Ixodes holocyclus* is a vector of the disease.

Siberian tick typhus. This is a relatively mild rickettsial disease, occurring in Central Asia and extending to the eastern coast of Siberia. The causative rickettsia is closely related to *R. conori*, but differentiable from it,²⁴ and has been called *R. sibericus*. The disease is believed to be transmitted by several ticks, including *Haemaphysalis concinna*, *Dermacentor sylvarum*, and *D. nuttallii*

RICKETTSIALPOX

Rickettsialpox was first observed in New York City in 1946, when 124 cases were reported, and subsequently has been found in urban areas on the eastern seaboard of this 840 RICKETTSIA

country. It has also been reported to occur in urban areas in Russia and has been isolated from wild rodents in Korea.²⁷ The disease is a mild febrile illness, characterized by a local lesion at the site of infection, and a varicelliform rash. It is rarely if ever fatal.

The causative agent is a rickettsia of the spotted fever group which is immunologically related to *R. rickettsii*, but may be distinguished from it; it has been given the name *R. akari*. The animal reservoir of infection is the wild house mouse, *Mus musculus*, and the disease is trasmitted by the mite *Allodermanyssus sanguineus*. The causative agent has been isolated from mice and mites as well as from patients' blood, and the human infection is apparently acquired

from the bite of infected mites. R. akari is pathogenic for both wild and laboratory mice and for the guinea pig. A fatal disease is produced in the mouse following intraperitoneal inoculation; the infection may be transmitted by spleen or brain, and the rickettsiae are demonstrable on microscopic examination of scrapings from the peritoneal cavity, as from the surface of the spleen. A febrile disease and scrotal reaction are produced in the guinea pig.

R. akari is readily cultivable in the embryonated hen's egg, in the amnionic cavity or the yolk sac. Yolk sac antigen fixes complement in the presence of patient's serum, but also cross-reacts with spotted fever

antibody.

Tsutsugamushi Disease (Scrub Typhus, Mite Typhus) 18, 40, 68

A disease of rickettsial etiology prevalent in the Far East is known as tsutsugamushi disease (dangerous bug disease), Japanese flood fever, or Kedani fever in Japan, as mite typhus in Sumatra, and as rural typhus or scrub typhus in Malaya. The last two are not to be confused with shop typhus of Malaya, which is murine typhus. The disease is very similar to spotted fever in its distribution and clinical picture but differs in the latter respect in that there is a primary sore and adenitis as in fièvre boutonneuse, and the symptoms include headache, orbital pain, a maculopapular rash, and fever. As in spotted fever, the case fatality rate varies widely, from 0.5 per cent to perhaps as high as 60 per cent in some areas such as Korea. The disease is characterized immunologically by a Weil-Felix reaction in which the O antigen of the OX-K strains of Proteus are agglutinated but the OX-19 strains are not.

The causative microorganism has been variously named R. tsutsugamushi, R. nipponica, R. orientalis, and R. akamushi; the first, R. tsutsugamushi, is the name commonly used. Strains are closely related immunologically but are readily differentiated by complement fixation. There seems, however, to be no clear serological differentiation into types, and the strains also differ in virulence and in degree of adaptation to egg culture. These rickettsiae are apparently quite unrelated to R. sennetsui, 4, 21 isolated in Japan from a glandular fever type of disease in man.

The strains commonly used as antigens

are the Gilliam and Karp strains, and sometimes the Seerengavee strain; serums from some cases are predominantly of one type or another, while others react to about the same titer with all three. The Gilliam strain is toxic to mice while the others are not, and the toxicity is not neutralized by antiserums to other strains of R. tsutsugamushi, or to only a slight degree. R. tsutsugamushi may be grown in the yolk sac or chorioallantois of the developing hen's egg, in tissue culture, and in the lungs of rats. Since this rickettsia occurs in the emulsion phase in ether extraction of yolk sac culture, the material may be purified by extraction before saline dilution or by differential centrifugation for the preparation of complement-fixing anti-

Experimental animals recovered from nonfatal infection show practically complete cross-immunity among strains of R. tsutsugamushi. Immunization with vaccine is a difficult matter, however, for apparently subcutaneous inoculation does not protect, but intraperitoneal vaccine protects against intraperitoneal challenge. Formalinized tissue culture vaccine or rat lung vaccine has been used for successful immunization of experimental animals. Killed vaccines are ineffective in man, but Smadel and his coworkers, 35, 64, 65 have produced an effective immunity with living vaccine given together with chloramphenicol to provide protection against infection. At present, however, active prophylactic immunization of man is not practical because of lack of cross-protection between the various strains of the rickettsiae.

Q FEVER 841

Tsutsugamushi occurs over a wide area in the Asiatic Pacific region, including Japan, Korea, Formosa, Indo-China, Malaya, Burma, Assam, New Guinea, and the Philippine Islands. It appears to persist as an infection of rodents and of mites and is transmitted to man by mites. The vertebrate host and insect host and vector differ somewhat from place to place. In Japan the rodent host is the field mouse, Microtus montebelli, and the disease is transmitted by the larvae of Trombicula akamushi. In Sumatra the vertebrate hosts are the house rat, Mus concolor, and the field rat, Mus diardii, and in Assam and Burma the wild rat, Rattus flavi-

pectus yunnanensis, and the tree shrew, Tupaia belangeri versurae, Except in Japan the most important insect vectors appear to be Trombicula deliensis, a mite very closely related to T. akamushi, Trombicula walchi which is regarded by some as identical with T. deliensis, and Trombicula fletcheri. The mites occur in low, damp areas of grass, underbrush, and scrub, in Japan along uncultivated banks of rivers, and the infection is thus restricted to certain localities. In the temperate climate of Japan the disease has a seasonal incidence, but in tropical regions there is no strict seasonal distribution of the disease in man.

Q Fever 39, 59

Q fever* was described in Australia in 1937 as occurring in slaughterhouse workers, and the causative agent was shown to be a rickettsia. What proved to be a closely similar, if not identical, rickettsia was isolated by Davis in the United States from ticks, and the disease it produced in guinea pigs was called nine-mile fever. When the Australian and American strains proved to be substantially identical, the disease was called American Q fever in this country, but, since the microorganism has been found to be of world-wide occurrence, the geographical designations have been dropped.

This microorganism is set apart from the other rickettsiae in a number of its characteristics. The American strains were found to pass bacteria-proof filters, and the rickettsia was named R. diaporica for that reason, but the name R. burnetii given the Australian strains had priority. It fails to form a toxin, is more resitant to heat and drying than the other rickettsiae, and is unique within the group, in that arthropod transmission is not an essential link in its dissemination. It is immunologically distinct from the other rickettsiae. In general, it appears to be sufficiently different from the other rickettsiae causing human disease to justify giving it independent generic status, and it is known as Coxiella burnetii.

Like the other rickettsiae, it grows profusely in the yolk sac of the embryonated hen's egg and may be cultivated in various kinds of tissue cultures. Guinea pigs may be infected, and the infection is either symptomless or accompanied by a febrile reaction, but there is a specific immunological response. There appear to be two kinds of antigens present in C. burnetii strains, distinguished by differences in the time required to reach peak titer of complement-fixing antibody in the infected person, and the strain is said to be in phase I or phase II;48 phase I antigen has higher protective potency as vaccine. 49 Complement-fixing antigen is grown in the yolk sac, and it is important to use a strain which will react with both kinds of antibody: the Henzerling and nine-mile strains have been used extensively for this purpose.

Disease in man. The disease in man is a febrile respiratory illness, with an incubation period of 14 to 26 days, and the infection is usually acquired by inhalation of infectious material. It characteristically takes the form of atypical pneumonia and is responsible for an appreciable portion of illness diagnosed clinically as primary atypical pneumonia, with pneumonic involvement demonstrable by x-ray examination in even mild cases. There is often evidence of liver involvement, headache is a predominant symptom, and there is no rash, but convalescence is apt to be protracted. Complement-fixing antibody is present in convalescent serums, and a hypersensitivity to intradermal vaccine may develop.29

The human disease is widespread, occurring in all major geographical areas, 10 as shown both by diagnosed illness and by the prevalence of serum antibody found in surveys. The largest number of cases has been reported from this country and various parts

^{*}The assumption that Q fever refers to "Queensland fever" is a misconception; in the original reports it refers to "Query fever."

842 RICKETTSIA

of Europe, especially in the Mediterranean area where the disease is known as epidemic hiberno-vernal bronchopneumonia or Balkan grippe, and occurred in Allied military forces in that area during World War II.

Infection in lower animals. The Australian studies showed the existence of a relatively intricate host-parasite relationship. There C. burnetii is maintained in wild animals, especially the bandicoot (Isoodon macrourus), and is transmitted by the ticks Haemaphysalis humerosa and Ixodes holocyclus. Cattle may be infected by these ticks, and in turn the cattle ticks Boophilus microplus and H. bispinosa may become infected. The rickettsiae occur in large numbers in the feces of infected ticks and survive for long periods in the dry state. The contamination of cattle hides with such infected feces, and the inhalation of this dust, is regarded as the probable mechanism of infection of slaughterhouse workers in Australia. The disease also occurs among slaughterhouse workers in this country, but the way in which the infection is contracted is uncertain.

Infection in dairy cattle is very widespread

over the United States, with herd infection rates ranging from 1 to 65 per cent.³⁷ C. burnetii is constantly present in the milk from such herds and is shed into the environment in infected placentas at parturition. Inapparent infection in dairy workers as reflected serologically is substantial, and parallels the incidence of infection in cattle.³⁸ Pasteurization of milk suffices to destroy the rickettsiae, but the margin of safety is very narrow, and the microorganism is occasionally found in pasteurized milk.¹⁷

The observations of Lennette and his colleagues have shown that in northern California the infection is maintained in sheep herds by contamination of the environment by milk and infected placentas taken at lambing time, and is reflected in a seasonal increase in human disease. It is probable that infection occurs by inhalation of contaminated dust. Similar observations have been made elsewhere. 42, 43, 66 There is also serological evidence 67 for the occurrence of reservoirs of infection in domestic fowl and wild birds, and their ectoparasites.

Trench Fever

In the course of the First World War a specific infection became known by the name of trench fever or Wolhynian fever. It is said to have caused almost one-third of all the illness in some of the armies in northern France and occurred also in Mesopotamia and Saloniki. It appeared again in the Second World War in the German army in Russia.28 It has a long incubation period (six to 22 days); the most constant symptom is pain in the legs; the fever is often high and of the relapsing type. Recovery is the rule. The disease can be transmitted to healthy men by the intravenous injection of whole blood taken from patients up to the fifty-first day of the disease.

Natural transmission is chiefly, if not solely, through the body louse. The bites of infective lice appear to be the most important element in producing trench fever, but the infectious agent is also present in the excreta and may enter through abrasions in the skin caused by scratching. The infectious agent is present in the urine of patients and is said to remain active for a long time in dry louse feces and dried urine.

The association of rickettsiae with the dis-

ease was established in 1919 by Arkwright and his co-workers on the basis of the infectiousness of lice, or louse excreta, containing very large numbers of the microorganisms following feeding on infected persons. The rickettsia, known as R. quintana or R. pediculi, has not been cultured, multiplying apart from man only in the stomach of the louse, and the disease has not been produced in experimental animals. Patients' sera agglutinate rickettsiae from the louse stomach, and diagnosis is based on the louse-feeding test, or xenodiagnosis.⁴⁴

In the original studies it was found that the blood remains infectious for lice, and the lice for human volunteers, for some months after disappearance of symptoms. Subsequently it has been found that the infection may persist for years, with relapse as long as 19 years after the initial illness, 45 which may be diagnosed by the louse-feeding test.

The causal relation of the rickettsia to the disease cannot be considered as fully established, but available evidence provides a strong presumption that such a connection obtains.

Laboratory Diagnosis of Rickettsial Disease³

Laboratory diagnosis is an indispensable adjunct to the identification of disease of rickettsial etiology and involves two general kinds of procedures; the one isolation and identification of the causative agent, and the other serological identification of serum antibody. The latter also allows retrospective diagnosis and estimation of prevalence of infection by surveys for antibody.

The isolation of rickettsiae is of limited diagnostic utility because of technical complications and inherent dangers. Guinea pigs and mice are the animals of choice and may be inoculated intraperitoneally with blood taken during the febrile stage of the disease. As described earlier, there may be little overt evidence of infection in the guinea pig with some kinds of rickettsiae, and blind passage, using spleen emulsion as the inoculum, may be required. In addition, blood may be taken and examined for serum antibody, indicating an immune response in symptomless infection.

The antibody response may be assayed nonspecifically by the Weil-Felix reaction, and specifically, using rickettsial antigen, by complement fixation, rickettsial agglutination, toxin neutralization except in the case of C. burnetii, and protection of experimental animals against known challenge inoculum. Of these, the last two are not practical for routine application, and by far the most commonly used serological reactions are the Weil-Felix reaction, which almost any laboratory may carry out, and the complement-fixation and/or rickettsial agglutination reactions. The first serves to identify or exclude the broad groups of rickettsiae but is diagnostic for the tsutsugamushi group; the complement-fixation reaction is group-specific but may be made specific or relative species titers may be diagnostic; and rickettsial agglutination is applicable in field studies and is regarded as more species-specific in some instances, e.g., the typhus fevers, than the unmodified complement-fixation reaction.

Weil-Felix reaction. The agglutination of OX-19, OX-2, and OX-K strains of Proteus allows provisional identification of the typhus groups of rickettsial disease but does not differentiate among the other rickettsial infections, and irregular results may occur within the so-called indeterminate, or Weil-Felix negative, groups of infections.

Normal serums from persons living in endemic localities often show an agglutinin titer of 1:50, and titers as low as this are regarded as negative, 1:100 as suggestive, and 1:200 as diagnostic. The Weil-Felix reaction shows an anamnestic response in a number of febrile diseases, especially typhoid fever. In typhoid fever, however, the titer of the Weil-Felix reaction does not continue to rise after the first week, whereas typhoid bacillus agglutinin continues to rise in titer. In rickettsial disease the agglutinin titer appears relatively early, reaching a titer as high as 1:400 by the end of the first week, and rising to 1:1600 or above by the end of the second week. It drops thereafter, falling to 1:400 or less by the eighth or ninth week after onset, and has no utility in retrospective sero-diagnosis. Though classified as an indeterminate type, spotted fever frequently shows a considerable agglutinin titer for Proteus, and differentiation from murine typhus may be difficult when the two diseases occur in the same area.

Specific serological reactions. Although in certain instances, notably tsutsugamushi disease, Proteus agglutination suffices; in general, complement-fixation carried out with purified rickettsial antigen is the serological method of choice, and rickettsial agglutination is equally specific.

In epidemic typhus fever, the complementfixing antibody titer shows an eight-fold rise in titer against the specific antigen between the first and third weeks, and is usually fourfold or greater than the titer observed to R. typhi antigen in unimmunized persons. Similarly, in murine typhus the specific complement-fixing antibody titer rises from less than 1:10 in the first week to 1:60 or more by the third week and is consistently higher than titers to R. prowazeki antigen in unimmunized individuals. Rickettsial agglutinin titers of 1:200 are regarded as diagnostic in both diseases. It may be extremely difficult to distinguish between the two by serological reactions when the disease occurs in previously immunized persons.23

The complement-fixation titer rises about eight-fold from the first to the third weeks of the disease in spotted fevers, to a titer of 1:80 or higher. A similar complement-fixing antibody titer rise occurs in rickettsialpox, but a positive Weil-Felix reaction is given with the OX-19 or OX-2 strains of Proteus

in spotted fever, but not in rickettsialpox. An approximately similar rise in complement-fixing antibody titer to *C. burnetii* occurs in Q fever, usually without confusing cross-reactions.

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Chapter Thirty-six

THE PSITTACOSIS— LYMPHOGRANULOMA VENEREUM GROUP OF MICROORGANISMS

The microorganisms making up the psittacosis-lymphogranuloma venereum group4, 69, 70 resemble one another closely, and it is believed that they may have differentiated from a common ancestral form by association with different host species. The group includes the causative agents of psittacosis and ornithosis, mouse pneumonitis, feline pneumonitis, human pneumonitis; organisms such as the Louisiana and San Francisco strains found in primary atypical pneumonia; the organism of opossum meningopneumonitis; and those causing bovine encephalitis and ewe abortion. The trachoma and inclusion conjunctivitis organisms are somewhat set apart from the group, although related to it, and are considered with it; the entire group is casually referred to as the PLT group of organisms.

The organisms of this group resemble the rickettsiae in a number of respects, i.e., they stain similarly and are sufficiently large that they fall just within the limits of optical resolution; there is some evidence that multiplication may include a process resembling binary fission; the diseases they produce may be treated effectively with certain antibiotics, and some strains are susceptible to the activity of sulfonamides. Similarities such as these have prompted some workers to group these microorganisms with the rickettsiae, but they are more appropriately regarded as another group of intermediate forms which constitute a link between the bacteria and the viruses. 55, 56 They are formally classified with the bacteria as members of the genus Chlamydia.59

These microorganisms occur in a variety of vertebrate hosts. Naturally occurring infections with the agents of lymphogranuloma venereum, trachoma, and inclusion conjunctivitis appear to be confined to man. Psittacosis occurs in psittacine birds, and the closely related, if not substantially identical, ornithosis occurs in wild birds such as pigeons and gulls, and in domestic fowl, especially the turkey; human infection is most commonly acquired by contact with such reservoirs of infection. Whether the pneumonitis agents of human origin, having minor but distinctive immunological character, occur primarily in man is uncertain. Pneumonitis infections in lower animals are relatively common; the infections are often latent, and it has been suggested that intensive study of apparently normal mice, birds, etc., may show the frequent presence of microorganisms of this kind.

Morphology and staining. The mature particle, or elementary body, is spherical or coccoidal in shape and relatively large, 200 to 300 m μ in diameter. The particle appears to consist of a central mass of electron-dense material surrounded by less dense material and enclosed within a limiting membrane. The structure is apparently not markedly rigid and tends to collapse on drying to give the appearance of a particle as much as 500 m μ in diameter. In shadowcast preparations the more rigid central mass is clearly apparent within the flattened particle. The membrane has been isolated and purified by lysis with desoxycholate and tryptic digestion, and found to resemble closely the cell walls of the gram-negative bacteria and rickettsiae in composition and properties⁴³ (Chap. Three).

Chemical analysis of purified elementary bodies of some of these microorganisms has shown that, like certain large viruses such as the poxviruses, they contain protein, lipid, carbohydrate, and both pentose and deoxypentose nucleic acids in proportions similar to those found in bacteria and rickett-siae.⁵⁴

Developmental cycle. These microorganisms occur in infected tissue as intracytoplasmic inclusion bodies or vesicles, and the morphology of these and the corpuscular elements making them up is variable as a consequence of the sequential occurrence of morphological types during the growth cycle as described elsewhere (Chap. Seven). In brief, following penetration of the host cell, the elementary bodies increase in size, to as much as 800 m μ in diameter, to become initial bodies which increase in numbers to form clusters or plagues in a ground substance or matrix. The corpuscular elements increase in number and decrease in size, giving rise to a vesicle containing large numbers of elementary bodies. In living preparations the particles are at first immobile, but as the vesicle matures the matrix apparently breaks down and the elementary bodies show brownian movement. Subse-



Figure 257. Shadow-cast electron micrograph of feline pneumonitis agent. Note the collapse of the spherical particle in the dried preparation showing the dense central mass and the outer limiting membrane. × 30,000. (Moulder and Weiss.)

quently the vesicle disintegrates, liberating elementary bodies which may then initiate a new infection. This growth cycle is completed in 24 to 48 hours, the time required depending on the strain of microorganism and the host tissue. Substantially this kind of growth cycle has been observed to occur with the meningopneumonitis, 37 psittacosis, 46 and trachoma^{2, 9} organisms.

Staining. The elementary bodies resemble rickettsiae in that they stain more readily with aniline dyes than do viruses, staining red with the Macchiavello stain and dark purple with the Castaneda stain. The relatively immature inclusion body, containing dense ground substance, is colored blue. As it matures the matrix becomes less dense, the larger particles are stained blue, and the smaller elementary bodies red by Macchiavello stain against the pale blue background of the matrix.

Toxin.¹⁹ All of the strains of lymphogranuloma venereum, psittacosis and ornithosis, and pneumonitis organisms examined have been found to be toxic. The activity is labile and destroyed in a short time at 37° C. or by low concentrations of formaldehyde. It is closely associated with the elementary body and would appear to be an endotoxin rather than an exotoxin. The toxicity is demonstrable by inoculation of mice with heavily infected tissue, commonly yolk sac of the infected embryonated hen's egg, in dilutions of 1:40 or less. Following intravenous inoculation with 0.5 ml. volumes death occurs usually within 24 hours. Because the toxicity has not been separated from living infective microorganisms, two-phase death curves may be observed, i.e., deaths attributable to toxicity occurring within 24 to 30 hours and those from infection beginning at about 48 hours. The common feature at autopsy is liver damage and often necrosis, but damage to the lungs and kidneys may occur also. The toxicity is specifically neutralized by antiserum (see below) but not by chemotherapeutic drugs.

Strains may vary considerably in toxicity. In a group of 39 strains, 22 of mammalian and 17 of avian origin, 17 of the mammalian strains killed in a dilution of 1:10 or greater, including two human pneumonitis strains that killed in a dilution of 1:80, while five of the avian strains failed to kill within 24 hours in dilutions of 1:10 or greater.⁴⁷ There is reason to believe that such toxicity may

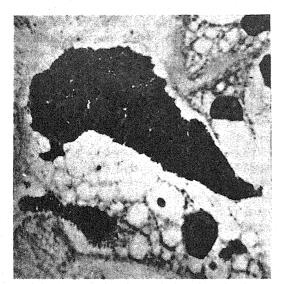


Figure 258. A mature intracellular vesicle of psittacosis in chick endodermal cell culture. May-Gruenwald-Giemsa stain; × 432. (Weiss.)

contribute significantly, as in the production of hemorrhage and edema in the lungs, to the pathogenesis of the diseases caused by these microorganisms.

Antigenicity. Complement-fixing. tective or neutralizing, and antitoxic antibodies are demonstrable. Two kinds of antigens, one heat-stable and one heatlabile, are concerned in complement fixation in the presence of convalescent or immune serums, and this reaction is useful for serodiagnostic purposes. Unfortunately, as usually carried out, it is nonspecific, not only with respect to microorganisms of the psittacosis-lymphogranuloma venereum group proper, but cross-reactions with these microorganisms are also given by the serums of persons infected with trachoma or inclusion conjunctivitis. With separation of the cell wall, it has been found that the group-specific complement-fixing antigens are intracellular, i.e., present in the lysate, while type-specific antigenicity occurs in the cell wall.44,62

The neutralization or protection test,¹⁸ carried out by incubation of the microorganism with antiserum prior to inoculation or previous passive immunization of the mouse respectively, was developed by Hilleman and by St. John and Gordon, and the toxin neutralization test by Manire and Meyer.⁴⁸ Only small amounts of protective and neutralizing antibody are found in con-

valescent serums so that such tests have no diagnostic utility. Hyperimmune serums from most laboratory animals are similarly deficient, but such antibody may be prepared to high titer in the rooster and used for differential purposes.³⁹

Both of these kinds of tests show a considerable degree of strain specificity. In a general way, infection experiments have suggested that the human pneumonitis strains are relatively specific, the strains of psittacine origin less so, and the meningopneumonitis (human) and pigeon strains have the broadest antigenic structures. More specificially, lymphogranuloma venereum, mouse pneumonitis, and feline pneumonitis agents have been found to be highly specific in the neutralization test, and, on the basis of antitoxic specificity. Manire and Meyer divided 27 strains into six groups, viz., (1) Louisiana (human) pneumonitis organisms, (2) San Francisco (human) pneumonitis organisms, (3) feline pneumonitis organisms, (4) meningopneumonitis (human) and certain ornithosis strains, (5) ornithosis strains isolated from pigeons, and (6) a group of strains of pigeon and human origin.

A hemagglutinin for mouse erythrocytes is formed by microorganisms of this group, allowing assay of hemagglutin-inhibiting activity.²⁴ It is not consistently present in infectious allantoic fluid, and is usually of low titer, 1:32 at the most, when present. It seems not to be eluted from mouse red cells, and the antigenicity appears to be nucleoprotein in nature and group-specific.²⁵

A passive hemagglutination test, using

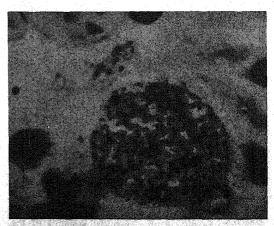


Figure 259. A vesicle containing elementary bodies from the lung of a mouse infected with mouse pneumonitis. Noble's stain; × 2100.

soluble antigen absorbed on tannic acidtreated sheep red cells, does not differentiate between ornithosis and pneumonitis -i.e., it is group-specific—but the sensitizing antigen is not identical with the complement-fixing antigen.

It is apparent that both common and specific antigens are distributed among these microorganisms and that different antigens are concerned in these tests. At the same time, neither the details of an antigenic structure nor the implications of host species on the immunological character of these organisms have been precisely defined.

Experimental infections. Differences in the pathogenicity of these microorganisms are evident in both the host range of naturally occurring infections and in the relative susceptibility of various experimental animals to infection. With the exception of trachoma and inclusion conjunctivitis agents, the mouse may be infected with all of the microorganisms of this group, and they may be grown in the embryonated hen's egg and various kinds of tissue culture.

Mice may be infected by the intranasal, intraperitoneal, and intracerebral routes to produce fatal infections with most strains of these organisms. Intranasal inoculation results in the development of a pneumonic process characterized by consolidation, and elementary bodies are found in large numbers in impression smears of the affected tissue. At autopsy animals infected by the intraperitoneal route show enlargement of liver and spleen and the presence of fibrous exudate in the abdominal cavity containing large numbers of elementary bodies. A meningoencephalitis with an exudate containing polymorphonuclear and mononuclear

cells is produced by intracerebral inoculation. Mice may also be infected by subcutaneous inoculation to give a generalized infection which is less often fatal and usually protracted.

These microorganisms may be grown in the embryonated hen's egg in the volk sac, where they multiply to high titer, and some grow well in the allantoic cavity or upon the chorioallantoic membrane. Multiplication of the feline pneumonitis agent in the yolk sac is associated with a high metabolic rate and an increased rate of synthesis of nucleic acid. According to Moulder and his colleagues⁵³ the energy for replication is supplied by high-energy phosphate bonds produced by the aerobic oxidation of endogenous substrates. Studies on the growth of psittacosis in chick embryo tissue culture^{3, 40, 50, 51} have shown that replication is dependent upon the presence of folic and/or folinic acid together with certain purines and amino acids. Although cytochrome C reductase activity1 and significant amounts of folic acid16 have been found in purified preparations of meningopneumonitis agents and in feline pneumonitis, mouse pneumonitis, and psittacosis agents, there is as yet no definite evidence that these microorganisms have a capacity for even the limited metabolic reactions demonstrable in rickettsiae.

Chemotherapy. 41 In general, tetracyclines effectively suppress the growth of these microorganisms, penicillin has an appreciable but lesser effect, and chloramphenicol and streptomycin are least effective. There is some variation among strains, and the San Francisco and Louisiana strains, and single strains of ornithosis agents of pigeon origin and of meningopneumonitis and feline pneu-

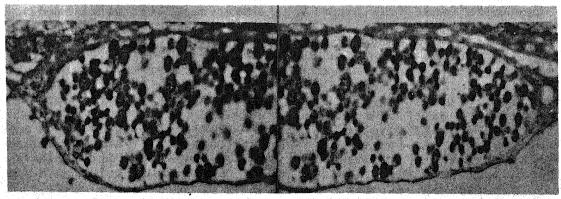


Figure 260. Electron micrograph of mature feline pneumonitis elementary bodies showing the two types of particles, the one smaller and dense and the other larger and granular in structure. × 15,000. (Litwin.)

monitis agents have been found to be unaffected by sulfadiazine.²⁹

The activity of these substances on these microorganisms is similar to their activity on sensitive bacteria, *i.e.*, they are primarily growth inhibitory. In the case of sulfonamides the activity is antagonized by paminobenzoic acid. Inhibition by penicillin and the tetracyclines is reflected morphologically as an abnormal growth; in the first instance irregular, vacuolated plaques are formed within the infected cell, and in the second multiplication of the initial bodies is inhibited.²⁶

Treatment of infections with these agents may result in an immune carrier state. The treated individual continues to harbor, or shed, the organism, sometimes for long periods, for example, in one human case of psittacosis for as long as eight years.

The growth-inhibitory effect of chemo-

therapeutic substances on the microorganisms of this group has been taken by some to imply the existence of as yet undetected independent metabolic activity. While this inference may eventually be substantiated by direct, and therefore more convincing, evidence, it is also possible that the effect is, as least in part, one upon the physiological mechanisms of the host cell concerned with replication.

Drug resistance. It is of particular interest that a number of these microorganisms may be made resistant to chemotherapeutic agents by passage in the embryonated egg or mouse in their presence. 27, 57 Such changes are presumably of the same nature as those observed with other microorganisms, and drug resistance has been used as a marker in genetic studies. 28, 58

Psittacosis and Ornithosis 49

Psittacosis, or parrot fever, was observed in Switzerland in 1880. For many years it was regarded as an unimportant disease, possibly caused by Nocard's bacillus, which is now designated Salmonella typhimurium. In 1929 and 1930 it appeared in many parts of the world as a disease acquired for the most part from South American parrots. The elementary bodies were described in 1930 independently by Levinthal in Germany, Coles in England, and Lillie in the United States, and for this reason are sometimes known as LCL bodies. Their nature and etiological relation to the disease were established in the early 1930's largely through the work of Bedson and his colleagues.

During that decade it also became apparent that exotic imported psittacine birds are not the only source of infection, and, in fact, that a domestic reservoir of infection occurs in gulls and other sea birds, pigeons, ducks, chickens, etc. The 1938 epidemic of pneumonitis in the Faroe Islands, for example, was associated with widespread infection in fulmar petrels. In the 1940's a variety of similar microorganisms was isolated from other lower animals and from human cases of pneumonitis.

The disease in man was originally called psittacosis because it appeared to be acquired largely from psittacine birds, and

the term ornithosis was introduced when the broader base of the reservoir of infection became apparent. The more inclusive term ornithosis, while not yet universally used, has gained increasing acceptance, especially for disease in, and acquired from, other than psittacine birds.

Pathogenicity for man. The disease produced in man by these microorganisms is essentially the same regardless of the source of infection. The incubation period is one to two weeks, and the onset may be sudden or insidious. The symptoms include chills and fever, photophobia, headache, anorexia, sore throat, nausea, and vomiting. A dry cough develops which persists and may become more severe, cyanosis and low blood presare frequent, and disorientation, apathy, insomnia, and occasionally delirium indicate involvement of the central nervous system. Leucocytosis does not occur until late in the disease or early convalescence, and the extent of the lung involvement is usually not apparent except by x-ray examination, which shows patchy areas of consolidation in one or both lungs. The organism may be found in the blood during the first week of the disease, and in the scanty sputum after the lungs are involved.

At autopsy the areas of consolidation are sharply demarcated from normal tissue;

alveoli containing air, serum, or serofibrous exudate, in which the cellular response is predominantly mononuclear, are dispersed in the areas of consolidation. The exudate cells and those of the hilar lymph nodes contain intracytoplasmic elementary bodies. Other necropsy findings include congestion and focal necrosis in the liver, possible enlargement of the spleen, cloudy swelling in the cardiac muscle, degenerative changes in the kidney parenchyma, and often congestion and edema of the brain and cord. Such findings are clearly indicative of the generalized nature of the infection. The case fatality rate was about 20 per cent in 1929 and 1930, dropped to about 10 per cent with the recognition of mild cases by serodiagnostic methods, and may be further reduced by therapy with tetracyclines.

Pathogencity for lower animals. Naturally acquired infection is not uncommon among certain lower animals and may occur, as a latent infection, even more frequently than has been supposed.

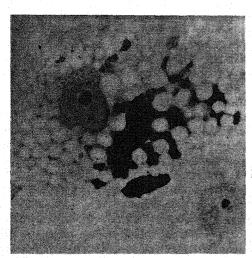
Birds. The natural hosts of these microorganisms among birds include parrots, parakeets, budgereegahs, etc., totaling not less than 31 species of the parrot family. Other birds such as canaries, finches, and sparrows contract the disease when exposed to infected psittacine birds; naturally occurring latent infection among pigeons appears to be relatively common, and microorganisms of the same group are found in doves, petrels, gulls, and egrets. The domestic chicken is naturally infected,

though not commonly, and spontaneous infection, possibly derived from wild birds such as gulls, and at times assuming epidemic form, occurs in turkeys. There are some differences in virulence and/or susceptibility, depending upon the strain of the organism and the host species. For example, strains of psittacine origin rarely produce fatal meningoencephalitis in pigeons, but those from pigeons and chickens usually produce fatal encephalitis on intracerebral inoculation.

In the parrot the disease is characterized by shivering, weakness, apathy and diarrhea, and respiratory disturbance may be evident. In these birds the disease is an infection of the liver and spleen with occasional involvement of the lungs, and the agent is present in the blood, affected tissues, nasal secretions, and feces. At autopsy of birds dying in the acute stage of the disease the spleen is enlarged and may show areas of focal necrosis, and the liver shows similar changes together with local hemorrhage and infarction, and a semipurulent exudate is found in the pericardial sac and on the air sac and inner lining of the sternum.

Naturally occurring infection in birds is frequently nonfatal, and there is reason to believe that there is a reservoir of latent infection of considerable proportions. Infected birds excrete the infectious agent in the droppings, and dried fecal material is an important source of airborne infection.

Mammals. A number of mammals are infected with closely similar microorganisms



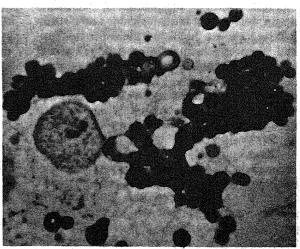


Figure 261. The effect of penicillin on the morphology of the feline pneumonitis organism. Left, untreated culture; right, penicillin-treated. The abnormal morphology in the presence of the drug is apparent, × 600. (Weiss.)

in a latent form, and such infections may be activated to give a clinically apparent, and possibly fatal, disease. This is a point of considerable practical importance in the isolation and study of organisms of this group. The meningopneumonitis organism, for example, which has been used extensively for experimental purposes, was originally isolated from ferrets inoculated intranasally with material from human pneumonitis; it is not clear whether the agent was present in the human disease and so isolated or whether it represented a latent infection in the ferret activated by the inoculation. Similarly, a number of these microorganisms have been found to occur as latent infections in mice.

Still other organisms of this group have been isolated from respiratory disease in man going under the names of virus pneumonia, pneumonitis, and primary atypical pneumonia. The majority of infections of this kind are of Mycoplasma etiology (Chap. Twentyseven) and are distinguished by the appearance of cold agglutinins in the serum, but some portion are of *R. burnetii*, miscellaneous viral, and other etiology.

Other mammals are naturally infected with microorganisms of this group, producing pneumonia of swine, meningopneumonitis of opossums, bovine encephalitis, ewe abortion, and cat distemper. The last, feline pneumonitis, has been widely used for experimental purposes. Of the usual experimental animals, the mouse is susceptible to infection with the organisms of this group by various routes of inoculation, the guinea pig gives little more than a febrile reaction to microorganisms of psittacine origin, but certain human strains and a strain from egrets are highly virulent for this animal. Some psittacine strains produce a fatal meningoencephalitis in the rabbit on intracerebral inoculation; rhesus monkeys may be infected by the intranasal or intracerebral routes, but rats are relatively resistant to intraperitoneal inoculation. In general, irregular results are obtained with the more resistant animals.

Immunity. As indicated above, the immune response results in the appearance of complement-fixing, protective, and antitoxic activity, varying with the host species. Complement-fixing antibody appears in 10 days to two weeks and hypersensitivity a week or two later. Effective immunity appears to be in part an infection immunity and dependent

upon the continued presence of the infectious agent. Some attempts have been made to produce a prophylactic immunity in man by the use of vaccines, but with as yet somewhat uncertain results.

Serodiagnosis. Serodiagnosis is dependent in practice on the complement-fixation reaction, carried out with paired serums to demonstrate a rise in titer. The rise occurs in 10 days to two weeks but may be delayed until two to five weeks by chemotherapy; a titer of 1:16, coupled with typical clinical findings, is often considered as positive.

The complement-fixation reaction is dependent upon one or the other of the complement-fixing antigens noted above. The heat-labile antigen is presumably protein in nature since it is inactivated by proteolytic digestion and tends to be strain-specific. and the heat-stable antigen, which is groupspecific, is considered to be polysaccharide because it is sensitive to periodate. The specificity of the test may be increased by preliminary absorption of the serum with steamed antigen and using living antigen in the complement-fixation test, but the use of living virulent antigen is impractical for routine purposes. Further differentiation between the two may be made on the basis of behavior of complement-fixing antibody titer; serums of persons infected with lymphogranuloma venereum organisms react to high titer with psittacosis antigen, but the titer declines during convalescence while that of psittacosis patients persists for many weeks or even months. As noted above, cold agglutinin is usually absent except in Mycoplasma infections.

The application of the complement-fixation to the diagnosis of avian infection is complicated in the case of chickens and ducks because immune serums from these birds do not fix complement in the presence of homologous antigen. A complement-fixation inhibition, or indirect complement-fixation, test, using heated antigen, has been devised⁴⁵ for use with such serums. In this reaction the fixation of complement by pigeon antibody in the presence of homologous antigen is specifically inhibited in the presence of antibody-containing serums of infected chickens or ducks.

Hypersensitivity. The hypersensitivity developed during infection has been useful for diagnostic purposes in surveys of flocks of domestic birds, especially turkeys. The antigen concerned is separable from com-

plement-fixing antigen by high-speed centrifugation, remaining in the supernatant. It may be extracted from infected yolk sac material with sodium lauryl sulfate, is precipitated by dilute acid, purified by butanol fractionation, differs from complement-fixing antigen in that it is soluble in 5 per cent phenol, and appears to be a lipocarbohydrate. Its activity is standardized in the guinea pig, and, inoculated intradermally into the wattles of turkeys, it gives the usual delayed type of hypersensitivity reaction which correlates well with the results of the complement-fixation reaction. 6

Laboratory diagnosis. Infection with these microorganisms may be established by isolation of the infectious agent from blood, sputum, or lung tissue taken at autopsy. The specimen is inoculated intranasally, intracerebrally, or intraperitoneally into mice and into the yolk sac of the embryonated egg. The microorganism is found as basophilic inclusion bodies and may be identified by serological methods.

Epidemiology. In the human disease the infectious agent usually enters the body via the respiratory tract. As indicated earlier, it is most often acquired from an animal reservoir of infection, usually indirectly by

inhalation of dried infectious material such as feces, and less commonly by direct contact with infected animals. Psittacine birds are the best known of these reservoirs, and infection appears to be common among wild parrots and parakeets, may assume clinical form in caged birds, and is generally endemic in aviaries and in breeding colonies of pigeons and ducks. The relative importance of mammalian, such as rodent, reservoirs of infection to the human disease is uncertain. Under some circumstances the infection may be transmitted directly from man to man as shown by a number of well-documented instances of infection of hospital or nursing personnel by patients. The highly contagious nature of the disease is evident from the number of laboratory infections which have occurred.

The incidence of the disease is largely an expression of risk. This factor accounts for its predominance in older women, and its tendency to occur as an occupational disease among professional handlers and breeders of birds. Control of the reservoir of infection in psittacine birds by quarantine has had some small success but in general depends upon avoiding sufficiently intimate contact with the animal reservoirs of endemic infection.

Lymphogranuloma Venereum

Lymphogranuloma venereum is a venereal disease caused by a microorganism closely related to the psittacosis-ornithosispneumonitis organisms described above. It is also known as climatic bubo, tropical bubo, lymphopathia venereum, and lymphogranuloma inguinale. The last is not to be confused with granuloma inguinale caused by Donovania granulomatis and discussed elsewhere (Chap. Twenty-seven). It was described as a clinical entity in 1913 by Durand, Nicolas, and Favre and is sometimes known as maladie de Nicolas et Favre. but it was not until the 1930's that its microbial etiology was recognized and the application of immunological methods allowed the grouping of various genital diseases such as elephantiasis of the female pudenda and inflammatory rectal strictures into a disease of common etiology.

The disease tends to occur in tropical regions, because of socioeconomic rather than climatic conditions, but its true in-

cidence is not known with any certainty because it is usually not reportable. In this country there appears to be a large reservoir of infection in the Negro as indicated by positive serological reactions in 25 to 40 per cent of those examined in certain areas.

The microorganism is morphologically indistinguishable from the others of this group and undergoes a closely similar, if not identical, growth cycle in the host cell. It is closely related immunologically through group antigens but is distinct in its pathogenicity, occurring apparently only in man as the natural host. While mice, monkeys, and embryonated eggs may be infected experimentally, this virus does not infect birds.

Pathogenicity for man.⁶³ Following the initial wide dissemination of the microorganism in the blood, spinal fluid, and other tissues, the primary lesion appears within a few days. It takes the form of a vesicular or herpetiform lesion occurring on the glans or prepuce of the penis, or on the posterior

portions of the labia, vaginal wall, or cervix, and may also be found within the urethra and in the anal region. The vesicle breaks down to leave a shallow ulcer that is not indurated and heals without scarring. This lesion is painless and is frequently overlooked.

The second stage of the disease is an invasion of the lymphatics. The regional nodes, commonly inguinal and also pelvic in the female, become enlarged and painful to form the bubo. In half or more of cases these suppurate, resembling similar lesions found in tuberculosis and syphilis, and may continue to drain for a long time. In this stage general as well as local symptoms occur, including fever and general aches, and in some cases arthritic and conjunctival symptoms and signs of involvement of the central nervous system may occur.

The third stage is an urethrogenitoperineal syndrome. The structural changes include nondestructive elephantiasis of the labia and clitoris in the female (esthiomene) and of the penis and scrotum in the male, and the rectum and anus become involved with the development of rectal stenosis, stricture, etc. The disease may be successfully treated with sulfonamides and the tetracyclines.³⁴

Pathogenicity for lower animals. The microorganism grows well in the yolk sac of the embryonated hen's egg. In mice inoculated intracerebrally leptomeningitis is produced, and the animals show incoordination, weakness, and paresis with a fatality

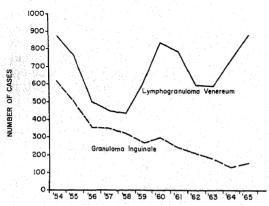


Figure 262. The incidence of lymphogranuloma venereum in the United States during the period 1954–1965 as indicated by the number of civilian cases reported. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U.S. Department of Health, Education and Welfare.)

rate of perhaps 30 per cent. On intranasal inoculation, pneumonitis, often fatal within a week, takes the form of a desquamative alveolitis with nodular inflammation around the capillaries and lymphatics, and the organism is demonstrable microscopically in the affected tissues.

The human disease is reasonably well reproduced in the monkey when inoculated into the prepuce, lymph nodes, or rectal tissue, with the development of typical inflammatory reactions. The monkey may also be infected by the intraperitoneal, intraocular, and intranasal routes, but not by intravenous or intracerebral inoculation.

Immunity. Effective immunity to this disease appears to be an infection immunity associated with continued presence of the infectious agent. Experimental inoculation of an infected individual gives a reaction similar to the Frei test (see below) but does not lead to the appearance of the initial vesicular lesion or an inflammatory response in the regional lymphatics.

The immune response is evidenced by the appearance of complement-fixing antibody in two to four weeks and the development of a hypersensitivity to the microbial substance. Both are useful for diagnostic purposes. The complement-fixation reaction is positive with serums from individuals infected with other microorganisms of the group (psittacosis, etc.), but may be made more specific by preliminary adsorption of the serum with heterologous antigen, and syphilitic serums may be nonspecifically positive. It is often not possible to obtain serum early in the disease, but a titer of 1:32 in conjunction with typical clinical findings is regarded as diagnostic, and an increasing titer has greater value.

Hypersensitivity was observed by Frei in 1925, and is demonstrable as a typical delayed reaction following intradermal inoculation infectious material. It was, in fact, the application of this reaction, the Frei test, that led to the identification of apparently diverse genital diseases, which had been known for many years, as manifestations of infection with this microorganism. The hypersensitivity develops one to six weeks after infection and remains positive apparently for the life of the individual, but transitory negative reactions may occur during sulfonamide therapy. The original Frei antigen was a 1:5 dilution of pus from a bubo in saline sterilized by heating. Mouse

brain antigens have not been satisfactory because of nonspecific reactions, and antigen prepared from the yolk sac of the infected egg is generally used. The skin test antigen is marketed under the name Lygranum. Cross-reactions³⁸ occur, but an acid extract of the microorganism appears to be more specific.

Epidemiology. The disease occurs all over the world¹⁷ and is found largely in ignorant persons of low economic status. It is usually, though not necessarily, trans-

mitted by sexual intercourse. Infection of the eye with an oculoglandular syndrome is known, and primary infection may occur in the mouth with painless vesicular lesions and swelling of the tongue, followed by infection of the glands of the neck. Occasional accidental infections occur, as in hospital orderlies infected while cleaning patients and in surgeons infected during removal of infected lymph glands. Control of the disease is difficult because it is not ordinarily reportable.

Trachoma and Inclusion Conjunctivitis 11,42

Trachoma and inclusion conjunctivitis are similar diseases of the external eye which are caused by microorganisms related to those of the psittacosis-lymphogranuloma venereum group. This subgroup is sometimes referred to as the TRIC (trachoma-inclusion conjunctivitis) group of agents. Naturally occurring infections are found only in man, and the host-specificity is sharp in experimental infections, which are similar to, but generally milder than, those in man, and can be produced only in certain primates.

TRACHOMA^{61, 64, 68}

Trachoma is an ancient disease and is referred to in the earliest known writings. It occurs in the Mediterranean area, especially in North Africa and the Middle East, in Russia, and the Orient, and in this country is found among mountain people in Tennessee, Kentucky, and West Virginia and among Indians on reservations. Its incidence is greatest, to as much as 90 per cent, in Egypt and the Middle East.¹⁰

There has been evidence of the microbial etiology of trachoma, and inclusion blennorrhea, since the work of the Trachoma Commission in the early 1930's. The reported isolation of trachoma agent in the embryonated egg by Tang et al.⁶⁵ in 1957 was subsequently confirmed by isolations in Africa,^{15, 66} in Taiwan, and in this country.³⁵ It may be isolated³⁰ and grown in cultures of human amnion cells, the McCoy cell line derived from human synovial tissue,⁶¹ and some strains may be adapted to HeLa cells,²²

as well as in the volk sac of the embryonated egg. It also produces a lethal pneumonic infection in mice following intranasal inoculation.32 In its morphology and other growth characteristics it resembles closely the other microorganisms of this group.³¹ It is related antigenically through group antigen but, when washed elementary bodies are used as complement-fixing antigen, the reaction is reported to be specific. 71 The elementary bodies contain a toxin, but it may not be antigenically homogeneous.⁵ The culture is susceptible to the broad-spectrum antibiotics⁶⁷ and reproduces the disease in various primates^{14, 20} with strain differences in virulence.

Pathogenicity for man. When the disease develops slowly, the first sign of infection is a slight ptosis of the lids, and there is a follicular hypertrophy of the upper tarsal conjunctiva. When the disease develops rapidly in a fulminating form, there is an inflammatory reaction characterized by a papillary or follicular hypertrophy of the conjunctiva and a mucopurulent exudate, and secondary bacterial infection is common, occurring in 50 per cent or more of cases. Intracytoplasmic inclusion bodies are found in the conjunctival and corneal epithelial cells in expressed follicular material and are most numerous in the cells from the superficial layers of epithelium from the upper tarsal and upper limbus areas.

The disease progresses with vascularization of the cornea and pannus formation and secondary cicatrization of the cornea, and partial or total blindness may result.

Microorganisms of this group also have been isolated from "nonspecific," i.e., non-

gonorrheal, urethritis.²¹ It may be successfully treated with sulfonamides or the broadspectrum antibotics.

There appears to be little or no effective immunity, since reinfection or relapse is not uncommon. Immunization with experimental vaccine, however, has been found to modify the experimental infection in human volunteers.³³ Infected individuals are negative to the Frei test but develop antibody which fixes complement in the presence of psittacosis and related antigens. The complement-fixation test is not sufficiently specific for diagnostic purposes; *i.e.*, it does not distinguish between trachoma and inclusion conjunctivitis.

INCLUSION CONJUNCTIVITIS

Inclusion conjunctivitis (inclusion blennorrhea, swimming pool conjunctivitis) is a benign conjunctivitis found in the newborn and in adults. It differs from trachoma in that pannus and scarring of the cornea are not observed; the disease is self-limiting and persistent chronic infection does not seem to occur although it may persist for some months, or even as long as a year in the adult.

The microorganism was isolated initially in the yolk sac of embryonated eggs by blind passage and eventual (ninth passage) adaptation to the egg,36 and subsequently elementary bodies were found at the first or second passage on primary isolation from the cervix of infected mothers and the eyes of infants.⁵² The culture produces a mucopurulent conjunctivitis in cynomolgus monkeys, and does not affect the cornea. It may be grown in cell culture¹³ and appears to be closely related in all respects to the trachoma agent. A preliminary study12 has suggested that an immunity effective under experimental conditions may be produced by vaccine.

Pathogenicity for man. In the newborn infant the incubation period is five to 12 days. The onset of the disease is sudden and characterized by an acute infiltration of the conjunctiva of the lower lid and a purulent exudate. The round cell infiltration results in a thickening of the conjunctiva, and the epithelium is infiltrated with polymorphonuclear cells and contains basophilic intracytoplasmic inclusion bodies indistinguishable from those of trachoma. The demonstration of these bodies, in epithelial

scrapings rather than exudate films taken for examination for bacteria, is diagnostic. Occasionally the disease is severe with the formation of transient pseudomembranes, but ordinarily it may be differentiated from gonorrheal ophthalmia on clinical grounds. The acute stage persists for perhaps two weeks and then gradually subsides, but the cornea does not return to normal for some months and may show evidence of infiltration for as long as a year.

The disease in the adult differs in that it is an acute follicular conjunctivitis with little discharge and a mild periauricular adenopathy. The follicles have the same appearance as those found in trachoma, but the hypertrophy is more marked in the lower lid, there is an absence of corneal change, and, on microscopic examination, the follicular material does not show necrotic changes. It may, however, be difficult to differentiate clinically from other forms of acute follicular conjunctivitis, and clinical diagnosis must be confirmed by the presence of inclusion bodies. The adult disease is also self-limited, resolving spontaneously without residual corneal or conjunctival changes, but tends to persist longer than in the infant.

Although overtly a disease of the eye, the infection is, in fact, one of the genitourinary tract and as such is transmitted venereally. Inclusion bodies are found in scrapings from the genitourinary tract of mothers of diseased infants, and the infection is apparently limited to the external os of the cervix and to transitional epithelium histologically closely similar to conjunctival epithelium. The infection is asymptomatic in the female but often produces a nonspecific urethritis in the male. Although of minor significance clinically, the genitourinary infection may be widespread.

The infant acquires the disease at birth from the infected mother, while the adult disease is most often acquired in swimming pools in which the water, which is not subjected to adequate chlorination or other treatment, is infected by persons having the genitourinary infection. Consistent with the nature of the reservoir of infection, inclusion conjunctivitis is an occupational disease of obstetricians and gynecologists. The serum of infected persons gives positive complement-fixation reactions with the group antigens and is not diagnostic for this disease. The disease in infants is not prevented by prophylactic silver nitrate, but

both the infant and adult diseases may be treated successfully with sulfonamides or tetracyclines, usually applied topically.

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Chapter Thirty-seven

THE VIRUSES OF EXANTHEMATOUS DISEASE AND THE TUMOR VIRUSES

A number of viral diseases of man and of lower animals are exanthematous in nature, and the viruses causing them have been said to be dermotropic because the predominant lesions occur in the skin. 131 Certain of these make up the more or less homogeneous group of poxviruses, having in common the properties of relatively large size, often just within the limits of optical resolution, and a brick shape, and occurring as intracytoplasmic inclusion bodies in the infected cells. Others, however, are spheroidal in shape, range in size from somewhat below the limits of optical resolution to one of the smallest of the known viruses, that of footand-mouth disease, and infected cells may contain intracytoplasmic or intranuclear inclusion bodies. They are heterogeneous and, with minor exceptions, as varieties

of herpesvirus, do not fall into groups.

While infection of the host cell with a virus usually results in relatively rapid death, a number of these viruses induce a proliferative response which may be relatively brief and followed by necrotic changes or sufficiently prolonged that hyperplasia is an outstanding feature of the disease. The proliferative response is apparent, though transitory, in smallpox, is more pronounced in molluscum contagiosum in man and in fibroma in rabbits, and in rabbit papilloma and myxoma the process is so marked that the virus appears to have carcinogenic properties. The viruses which induce a pronounced proliferative response are sometimes grouped as the tumor viruses; such a group is heterogeneous; e.g., it includes both pox-like and smaller spheroid viruses.

The Pox Group of Viruses^{53, 58}

The viruses making up the pox group resemble one another morphologically and in the pathogenesis of the diseases they produce, and they are immunologically related to one another. They are differentiated by host specificity and relative pathogenicity. The group includes the virus of variola which occurs in man and that of vaccinia, together with the viruses causing a group of pox diseases of lower animals. The viruses of molluscum contagiosum and

of rabbit fibroma and myxoma are closely similar morphologically but tend to be set apart from the pox group on the basis of the diseases they produce. They are considered with the tumor viruses.

Morphology. The viruses of this group are among the largest of the viruses, and the elementary body is just within the limits of optical resolution. The higher resolution made possible by electron microscopy has made it clear that, at least in many prepara-

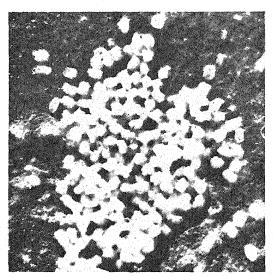


Figure 263. Electron micrograph of thin section through the chorioallantoic membrane of a 10-day chicken embryo 24 hours after inoculation with vaccinia. The numerous elementary bodies can be seen to possess a cuboidal or cylindrical shape. × 15,000. (Wyckoff: Zellforsch.)

tions suitable for examination by this method, the virus particle is a regular sixsided structure with rounded corners often described as brick-shaped, with dimensions of 200 to 250 m μ by 250 to 350 m μ . In some preparations the bodies appear to be ovoid in shape and in either case contain a central mass of electron-dense material.

These viruses occur as intracytoplasmic inclusion bodies known as Guarnieri bodies in the case of variola and vaccinia and as Bollinger bodies in fowlpox. These bodies differentiate into, and in mature form are composed of, elementary bodies called the Paschen bodies of variola and vaccinia and Borrel bodies of fowlpox. Both inclusion bodies and elementary bodies may be stained with polychrome stains such as Giemsa, by steaming with bicarbonate-crystal violet, and other methods, but do not stain satisfactorily with the usual bacterial stains.

Chemical composition. Like the rickettsiae and the psittacosis-lymphogranuloma venereum group of organisms, the chemical composition of the poxviruses is similar to that of bacteria, including nucleic acid in amounts of 6 to 7 per cent in contrast with the large amounts found in some other viruses, together with protein, polysaccharide, and lipid. The nucleic acid is DNA, predominantly double-stranded, and makes up the central core. Highly purified preparations have been found to contain copper and flavin together with biotin. If these are to be regarded as integral parts of the virus, they may represent rudimentary or vestigial fragments of a respiratory mechanism, but it is at least equally possible that such substances represent contamination originating in the infected tissue. The ether-resistance of the poxvirus group as a whole is variable, but the viruses considered here are ether-resistant.

Viral replication appears to Growth. occur in the cytoplasm of the infected cell. Following entry of the virus particle into the host cell, a portion of the cytoplasm surrounding it differentiates to form a matrix. When the matrix is dense it is the inclusion body, but viral replication may occur in a matrix that is not sufficiently dense to appear as an inclusion body. The matrix subsequently breaks down into elementary bodies, which may be in various developmental stages as described elsewhere (Chap. Four), and eventually the mass breaks down completely, and the cytoplasm of the cell is filled with elementary bodies which may then infect other cells.

Antigenicity. At least five kinds of antigens are present in the viruses of this group, certain of which are much better known in some of the viruses than in others. The LS soluble antigen of variola and vaccinia, found free in infected tissues, appears to be a single complex of which the L antigen is heat-labile and the S antigen is heat-stable. These antigens are demonstrable by complement fixation. A nucleoprotein (NP) antigen is present also, and these viruses form a hemagglutinin which agglutinates fowl erythrocytes, and in the case of mousepox mouse erythrocytes also, which is separable from the elementary body and associated with particles about 65 mu in diameter. The hemagglutinin activity is specifically inhibited in the presence of antibody.

During the replication cycle of the virus in HeLa cell culture these antigens appear in sequence. The LS antigen is detectable in about four hours, and the NP antigen in five to six hours, both in the cytoplasm of the infected cell and preceding the appearance of the infective particle. The hemagglutinating antigen is apparentely a by-product of virus-cell interaction and not found until about 10 hours postinfection.⁷²

None of the antibodies to these antigens

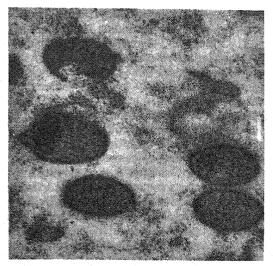


Figure 264. Electron micrograph of vaccinia virus in a section of infected chorioallantoic membrane showing the oval shape observed in some preparations. Note the small mass of electron-dense material in two of the virus particles. × 53,000. (Morgan: J. Exp. Med.)

is associated with neutralization or protection; rather, the ability to stimulate protective antibody formation is associated with infectivity of the virus strain, and it appears to involve antigens other than those just described.⁴

Pathogenesis of pox disease. 85 Although there is a considerable degree of host specificity among these viruses, differences in the pathogenesis of the diseases they produce may be considered to be variations on the same theme. The proliferative response alluded to above, for example, is minor in variola and vaccinia, but in some forms of fowlpox so marked that the disease has been known as contagious epithelioma.

The viruses may be grouped on the basis of tissue tropisms, e.g., the poxviruses are dermotropic, and such tropisms are inferred from the site of the overt or predominant lesion. It is significant, however, that viral replication may occur without obvious damage in cells and tissues apart from those in which the predominant lesions occur, and that such replication and dissemination may be an integral part of the pathogenesis of the disease. The occurrence of viremia, for example, exposes the virus to the action of humoral antibody and is fundamental to effective immunity to such a disease. In the case of mousepox the sequence of events has been worked out in detail by Fenner³⁵ (similar results have been obtained with rabbitpox¹²), and it is a fair presumption that this disease may be taken as a prototype, for the present at least, of similar pox diseases such as variola and vaccinia.

The incubation period of mousepox is about seven days, and by 11 days after infection the disease has progressed to severe rash with ulceration. Following initial infection, the virus multiplies in the skin and spreads to the regional lymphatics and multiplies there within the first two days of the incubation period. The virus is then shed into the blood stream to give primary viremia in the second or third day, spreading the infection to the spleen and liver, where it multiplies further and produces necrosis. Virus is again shed into the bloodstream to give secondary viremia on the fourth and fifth days, spreading the infection to the skin to set up foci of infection on the sixth day. By the seventh day, the end of the incubation period, the primary lesion, a swelling of the footpads, occurs; a papular

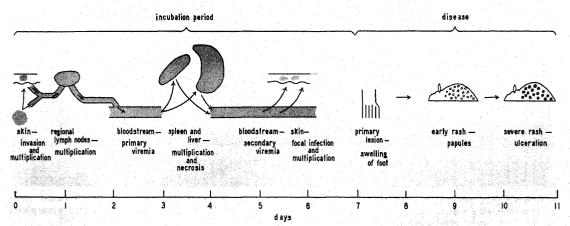


Figure 265. Diagrammatic representation of the pathogenesis of mousepox infection. (Redrawn from Fenner.)

rash appears by the ninth day which progresses to severe rash and ulceration by the eleventh day. This sequence of events is illustrated diagrammatically in the accompanying figure. Clearly, during the incubation period the virus is widely disseminated in the body, multiplying in cells not concerned in the primary lesion of the disease. As will appear (Chap. Thirty-nine), analogous phenomena occur in infections with other kinds of viruses also.

VARIOLA AND VACCINIA

Smallpox (variola) is an ancient disease which is known to have occurred in epidemic form in China as early as the twelfth century B.C., and is present in endemic, and from time to time epidemic, form in Asia, Africa, and the Middle East. It was widely disseminated in Europe during the Crusades, and was apparently introduced into the Western Hemisphere in the early sixteenth century, spreading into Central and South America, and new infection continued to be introduced by the slave trade.

This disease occurs in two forms differing markedly in severity. The one, variola major (malignant smallpox, black smallpox, etc.), is characterized by a case fatality rate of 25 to as much as 40 per cent. The milder form, variola minor, has a case fatality rate

of perhaps 1 per cent, and has been given a variety of names including alastrim, Kaffir pox, cottonpox, Cuban itch, and paravariola. The causative viruses of the two forms of the disease are almost indistinguishable except for a slightly greater virulence of variola major for the chick embryo. This difference in virulence may be enhanced by varying the temperature of incubation to give a laboratory test.²⁹ The diseases are differentiated on epidemiological grounds, *i.e.*, on the basis of the case fatality rate.

In many areas, including the United States, variola minor has tended to displace the malignant form of the disease. At the present time Asia and Africa are the main centers of variola major infection, with India the greatest single source of cases, and this form of the disease is endemic in Portugal in Europe and in Mexico in North America. 89 The introduction of variola major from endemic centers of infection remains a constant possibility; for example, this occurred in New York City in 1947. In this country the number of cases of smallpox has declined from the 30,000 to 50,000 cases reported per year in the 1920's to no confirmed cases being reported since 1954.

Vaccinia is almost always a purposefully acquired infection to produce immunity to smallpox (see below), and its occurrence in this way dates from Jenner's observations in the latter part of the eighteenth century.

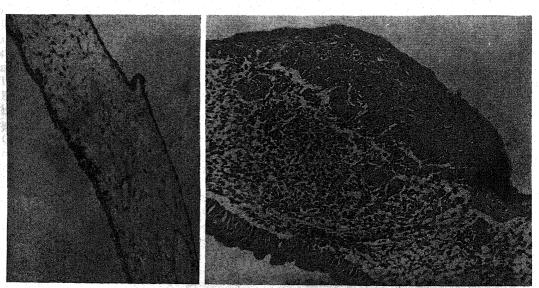


Figure 266. Sections of chorioallantoic membranes of 13-day embryonated eggs. *Left*, uninoculated; *right*, section through a variola lesion. Note the thickening of the membrane and discrete area of epithelial hyperplasia. × 100. (U.S. Army photograph, courtesy of Dr. N. Hahon.)

VARIOLA 863

Vaccinia is almost always a mild, localized disease in man; generalized vaccinia occurs rarely in the normal individual, but may occur when a nonimmune child with previously existing skin lesions, e.g., eczema, acquires vaccinia by contact.

Smallpox in man.²⁴ Variola virus is resistant to drying, and the crusts from pustules are highly infectious. Human infection is acquired by contact, direct or indirect. with such infectious material. There is reason to believe that in the typical disease primary infection occurs in the respiratory tract with multiplication of the virus in minimal, often but not necessarily noninfectious. lesions.28 The virus spreads to the regional lymphatics and into the blood stream to the viscera, where it multiplies during the latter part of the incubation period of about 12 days. When virus is released from these sites to give secondary viremia, symptoms, including chills, fever, prostration, headache, backache, and vomiting, appear, and the virus may be found in the blood. The secondary viremia gives rise to foci of infection in the skin, mucous membranes, and viscera, and the skin lesions appear. These occur as a single crop and are at first macular, and then pass through papular and vesicular stages to the pustule over a period of five to 10 days.

Histologically there is proliferation in the prickle-cell layer of the skin with the appearance of cytoplasmic inclusions. Intracellular edema, evidenced by the appearance of vacuoles, occurs and is followed by intercellular edema. The vacuoles enlarge, become confluent, the cell membrane is distended and breaks down, and vesicles are formed by the coalescence of adjacent affected cells. The epidermal reaction is followed by dilatation of blood and lymph vessels, involvement of the basilar layer, the appearance of an inflammatory exudate, and necrosis in the corium which leads to the typical scarring.

Chemotherapy. The most common complication is pyogenic infection, and antibiotic therapy is effective in minimizing such infection with consequent reduction in

N-methylisatin β-thiosemicarbazone

residual scarring, although chemotherapeutic agents are ineffective against the virus infection.

 β -thiosemicarbazone and some Isatin closely related compounds have been found to have antiviral activity in experimental poxvirus infections. This compound and the methyl derivative is effective against experimental variola, vaccinia, and cowpox as a prophylactic agent but inactive against mousepox, while the reverse holds true with isatin β -dialkylthiosemicarbazone.⁸ Nmethylisatin β -thiosemicarbazone has been tested in man and found to have marked prophylactic value in contacts.9 The nature of the antiviral effect is not understood, but it appears to be an interference with maturation of the virus particle.

Immunity. Smallpox has been the classical example of infectious disease in which recovery is associated with the development of a highly effective and long-lasting immunity, and there is no doubt that the individual remains immune for many years. The immune response is evident in the appearance, during the course of the disease, of complement-fixing and hemagglutinininhibiting antibody activity, but, as indicated earlier, these antibodies do not appear to be associated with effective immunity. Protective antibody is formed which, unlike complement-fixing and hemagglutinin-inhibiting antibodies that decline to insignificant levels in some 12 months, is demonstrable for years, and may be demonstrated by appropriate tests such as tissue culture assay.20

There is a high degree of cross-immunity among the various kinds of poxviruses which is demonstrable in experimental animals. That between variola and vaccinia is of a high order and allows the use of the latter mild infection to produce an effective prophylactic immunity to smallpox in man (see below).

Laboratory diagnosis. The laboratory diagnosis of smallpox assumes significance in the prompt and accurate identification of atypical cases in which the disease is modified by prior vaccination, and to exclude generalized vaccinia, varicella (see below), etc. It is dependent upon the demonstration of the presence of the virus, either directly or inferentially from the presence of specific antibody.

Inclusion bodies are found in stained smears from lesions in the papular and vesicular stages of the disease, and virus may be isolated from these lesions on the chorioallantoic membrane of the 12- to 14-day-old embryonated hen's egg. Specific antigen is demonstrable in the lesions by complement fixation in the presence of known antiserum.

The most readily applicable diagnostic tests are those for serum antibody, complement-fixing antibody, and hemagglutinininhibiting antibody. The former may not appear until the later eruptive stage of the disease. It has been reported that hemagglutinin-inhibiting antibody is detectable even in the pre-eruptive stage of the disease, reaching titers of 1:2000 by the end of the first week. Antibody titer is of questionable diagnostic significance in recently vaccinated persons, but a rising titer during the course of the disease may be regarded as diagnostic.

Vaccinia and immunization.^{13, 23} Antibody to the LS and NP antigens is produced by inoculation with noninfectious material, but it has not been possible to produce an effective immunity to smallpox by the use of inactive virus. The development of such an immunity appears to be dependent upon infection with variola or related viruses of which vaccinia is the most important.

The practice of variolation, deliberate inoculation of man by application of the dried crusts of variola pustules to the skin or nasal mucous membranes, or by ingestion, was common in the Orient in ancient times. It was introduced into Europe in 1718 by Lady Mary Wortley Montagu, wife of the British ambassador at Constantinople. The infection so produced was usually much less severe than naturally acquired smallpox, and the appreciable mortality rate considerably less than that of the natural disease.

Meanwhile it had been observed by a number of persons that those who contracted cowpox from infected cattle did not acquire smallpox upon subsequent exposure. Cowpox is an exceedingly mild disease in man, rarely if ever fatal, in which the lesion is almost always limited to the site of inoculation. These casual observations were extended by the Englishman Jenner, who published his classic treatise on the relation of the two diseases in 1796. Prophylaxis of smallpox by inoculation with cowpox, or so-called Jennerian prophylaxis, was favorably received and widely applied.

Vaccinia virus. It was assumed by Jenner that cowpox virus is variola virus adapted to the bovine host. The cow may be infected

with variola, at first with insignificant lesions following intracutaneous inoculation, but on passage in this animal a vesiculopustular eruption is produced which is similar to, but not identical with, that of naturally occurring cowpox. While variola, vaccinia, cowpox, and mousepox are closely related immunologically, antigenic differences may be distinguished by cross-neutralization tests. In this respect vaccinia is found to be more closely related to variola than to cowpox, and in turn cowpox is more closely related to mousepox than to variola.37 This suggests that present strains of vaccinia virus were derived from variola virus rather than cowpox virus, but it has not been possible to derive them experimentally.54 In this connection it is of interest that naturally acquired cowpox in man is a somewhat more severe disease than vaccinia, and that in recombination experiments with vaccinia variants in mixed infections in HeLa cell culture and on the chorioallantoic membrane, the recombinants, apparently analogous to bacterial recombinants, tended toward lesser virulence.38

Vaccinia in man. Human inoculation with vaccine lymph (see below) is usually intracutaneous by the so-called multiple pressure method in which 30 to 40 punctures into the skin are made with a needle through a drop of vaccinia virus preparation about 1 cm. in diameter. After three or four days a papule appears which becomes vesicular on the sixth or seventh day, progresses to a pustule at 10 or 12 days, and then regresses. Fever may occur from the fourth to eighth day, and there may be some tenderness and enlargement of the regional lymph nodes. The crust which forms on the pustule becomes detached about three weeks after inoculation (foveation), leaving a red pitted scar which turns white in time. Complications are quite rare. Generalized vaccinia, with eruption at other than the site of inoculation and a more pronounced general reaction, may occur when other skin lesions, such as eczema, are present at the time of inoculation. Postvaccinal encephalitis, which is extremely rare, is similar to that following the inoculation of other immunizing preparations.52

The foregoing reaction is that given by a fully susceptible individual and is sometimes called the primary reaction. It is modified in the partially or fully immune individual to give a vaccinoid reaction in the first in-

VACCINIA 865

stance and an immune reaction in the second. The vaccinoid reaction is similar to the primary reaction but accelerated and less severe. While there are gradations in the response, in general the papule appears at two days, and the maximum reaction is reached between three and seven days. The immune reaction consists of the development of a papule or shallow vesicle which reaches its maximum size in eight to 72 hours, disappears without going through a pustular stage, and leaves no scar.18 The immune reaction is given by inactivated as well as active virus and represents an allergic response to the vaccine material. It is of practical importance to emphasize that a vaccine giving the immune reaction is not necessarily active and therefore may not be an effective immunizing agent for the nonimmune.

Experimental Infections. Variola virus infects only man and monkeys but is readily cultivable on the chorioallantoic membrane of the embryonated hen's egg, where it produces pock-like lesions, and it may be maintained in serial passage in the yolk sac. The disease in monkeys⁴⁸ is similar to that in man, and there is some reason to believe that natural infection may occur. Only minimal lesions are produced in the rabbit and calf. It is extremely difficult to pass the infection serially in the rabbit, a characteristic that differentiates variola sharply from

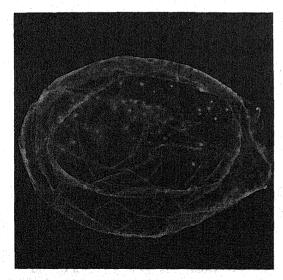


Figure 267. The gross appearance of the pock-like lesions produced by variola virus on the chorioallantoic membrane of the embryonated egg after 72 hours' incubation. Hematoxylin and eosin; × 100. (Hahon and Ratner, J. Bacteriol.)

vaccinia, and on serial passage in the calf the virus loses its original character and becomes vaccinia-like. The adult mouse may be infected by intracerebral inoculation but massive doses are required to produce regular death patterns. ¹⁴ The suckling mouse appears to be more susceptible and offers possibility for the assay of infectivity and protective antibody. ⁷⁷

In contrast, most laboratory animals may be infected with vaccinia virus, and it is readily cultivable on the chorioallantoic membrane of the embryonated egg, where it produces pocks closely similar to those of variola. The rabbit is the usual experimental animal, and infection is produced by all the customary routes of inoculation. The virus adapts relatively readily to growth on various kinds of tissues, and coincident alterations in virulence have resulted in strains which produce encephalitis, orchitis, or pneumonia, and strains differ in other respects such as pock morphology and heat resistance.

Vaccine for human use is usu-Vaccine. ally produced in the calf, though other animals, such as sheep, may be used also. The inoculum, or "seed" virus, is maintained at maximal activity by frequent passage in the rabbit and is used to inoculate the shaved abdomens of calves. After confluent vesicles have appeared along the scratches, the virus is harvested by scraping off the vesicle walls and contents, and this material, vaccine lymph, is emulsified in glycerol. The activity is stable for very long periods when vaccine lymph is stored at - 10° C., for about six months at 0° C., but for little more than a week at room temperature.

The virus may also be grown for vaccine purposes on the chorioallantoic membrane of the chick embryo, or in chick embryo tissue culture. Since the virus is grown under sterile conditions, problems of bacterial contamination, e.g., tetanus bacilli, etc., that are inherent in calf lymph vaccine production do not arise. These culture vaccines do not seem to be as effective immunizing agents as the calf lymph preparation, possibly because of adaptive changes to chicken tissue.

The activity of vaccines is assayed by the rabbit scarification test, 65 i.e., initial infection but not serial passage, but it has been urged by some that potency may be assayed equally, or possibly more, reliably by tissue culture methods 7, 19, 66 or by pock

counts on the chorioallantoic membrane of the embryonated egg.⁹⁶ Vaccine potency is referable to an international standard.⁶⁷

POX DISEASES OF LOWER ANIMALS

Pox disease occurs in a number of lower animals, including cattle, mice, and fowl, as noted earlier, and in addition in sheep, horses, swine, and a number of birds such as pigeons and canaries. In general, these diseases are characterized in the acute form by a vesicular eruption, but lesions may occur in the mouth as in stomatitis of horses, or the proliferative response to the infection may predominate as in one of the clinical types of fowlpox. In all of these diseases inclusion bodies of the Guarnieri type are found, often in large numbers, in the affected epithelial cells, and the causative viruses are morphologically closely similar and immunologically related to one another. Not all are equally well known, and only three, cowpox, mousepox, and fowlpox, will be considered here.

Cowpox. Cowpox is a mild, naturally occurring disease of cattle, characterized in the acute form by a vesicular eruption on the udder and teats. It is not a common disease, at least in the acute form. It is transmissible to man, as noted earlier in connection with Jenner's work, and the human disease is somewhat more severe than vaccinia. The virus is distinguished from variola and vaccinia in its hemorrhagic properties, apparent in the pocks formed on the chorioallantoic membrane of the chick embryo. and the vesicle fluid in bovine and human infections may contain blood. There is a cross-immunity between cowpox on the one hand and variola and vaccinia on the other, although minor antigenic differences are demonstrable. Cows may also acquire vaccinia from man.

Mousepox.³⁶ Mousepox, or ectromelia, is a naturally occurring infection of laboratory mice which was described as of viral etiology in 1930, and its relation to the pox diseases was demonstrated in 1945. It occurs in many parts of the world but does not seem to be present in this country as a naturally occurring infection, at least in the acute form. This disease has been particularly useful as a model experimental infection, such as for the study of the pathogenesis of pox disease, as described earlier, and as an immunizing infection comparable to

smallpox or diphtheria in experimental epidemiological studies (Chap. Ten).

The primary lesion develops at the portal of entry but is preceded by extensive multiplication of the virus and necrosis in the viscera, the spleen and liver particularly. In the acute form of the disease death occurs within two or three days after the appearance of the primary lesion, and on autopsy areas of necrosis are found in the liver and spleen. If the animal does not die at this point, a rash develops, at first papular and then vesicular with ulceration, and when the primary lesion is in the footpad, as is often the case, the tissue becomes edematous, then gangrenous, and sloughs off. The mortality rate is often high, but strains of the virus vary in this respect, and the infection may persist in latent form. The large cytoplasmic inclusion bodies are numerous in affected epithelial cells.

The virus produces only a latent, inapparent infection in the rat, it may be grown on the chorioallantoic membrane of the embryonated hen's egg, and rabbits and guinea pigs can be infected with egg-passage strains. It is immunologically related to variola and vaccinia and to cowpox.

Fowlpox. Fowlpox is a disease of domestic fowl which occurs in three clinical forms, *viz.*, the form in which the proliferative response results in the development of epithelioma-like growths on the comb, wattles, and skin of the head; the form in which yellowish membranous lesions are found in the mouth; and a form characterized by the presence of a watery or mucopurulent discharge from the nose and eyes known as roup.

The virus is morphologically similar to the other poxviruses, but somewhat larger than vaccinia and variola viruses. The virus particles, or elementary bodies, are known as Borrel bodies in this disease, and the large intracytoplasmic inclusion bodies are called Bollinger bodies. It is closely related to, but probably not identical with, the viruses of canary pox and pigeon pox. A variety of domestic birds may be infected experimentally, including the turkey, pigeon, goose, duck, and guinea fowl, together with a number of wild birds such as quail and pheasant. This virus, or closely related group of viruses, is set apart from the other poxviruses in that it is not pathogenic for mammals such as man, goat, sheep, mouse, rat, or guinea pig, but it is cultivable on the chorioallantoic membrane of the embryonated hen's egg.

The Herpesviruses

The group of agents considered to be herpesviruses characteristically proliferate in the nuclei of infected cells with the production of intranuclear acidophilic inclusion bodies. At one time the name Nitavirus (Nuclear Inclusion Type A) was suggested for this group, but has been discarded. The virus particle has cubic, icosahedral symmetry, the nucleocapsid is 95 to $105 \text{ m}\mu$ in diameter, and the particle is considerably larger, 180 to $250 \text{ m}\mu$, enclosed in its envelope. These viruses are ether-sensitive DNA viruses.

They fall into two subgroups, designated A and B:82 those of subgroup A are readily released from infected cells, while those of subgroup B are more closely cell-associated. Subgroup A includes the virus of herpes simplex (Herpesvirus hominis) occurring in man; that causing a naturally occurring disease of swine called pseudorabies or "mad itch;" a virus known as B virus found in monkeys which is immunologically related to both herpes and pseudorabies viruses; and virus III which occurs in rabbits. Subgroup B is made up of the varicella (chickenpox)-zoster virus found in man, and the cytomegaloviruses (salivary gland disease viruses), which occur in both man and lower animals.

HERPES SIMPLEX¹¹⁰

Herpes simplex (herpes febrilis, herpes labialis) is a naturally occurring disease of man characterized by vesicular lesions in the epithelial layers of ectodermal tssue and the presence of intranuclear inclusion bodies in the affected cells. The virus is a single entity with possibly minor variations in antigenic character between strains that do not seem to be reflected in the epidemiology of the disease. In the infected chorioallantoic membrane, the virus first appears as a small particle, 30 to 40 m μ in diameter, within the nucleus. These increase in size, apparently in part by the acquisition of a membrane, escape into the cytoplasm and acquire a second membrane, and finally emerge as mature particles.¹³² The presence of the virus in both nucleus and cytoplasm of infected cells has also been demonstrated by the fluorescent antibody technique.⁷⁰

In cell culture two general types of cytopathic effect are produced, (a) that characterized by the formation of syncytial giant cells, and (b) proliferative changes with the rounding and piling up of cells. These appear to be characteristics of strains which may be separated from one another when they become admixed. These varieties are substantially identical with respect to antigenicity and other biological properties but are reported to differ slightly in density as indicated by small differences in sedimentation rates. They are known as the GC, or giant cell, and P strains.

The disease in man. The vesicular lesions of herpes begin as a local burning sensation, followed by the appearance of papules which become vesicular and coalesce to form groups of thin-walled vesicles. The prickle-cell layer is affected with intra- and intercellular edema, and there is capillary dilatation and infiltration of inflammatory cells in the corium but no necrotic changes. The vesicular fluid contains epithelial cells and multinucleated giant cells with inclusions present in nuclei, and the fluid is infectious. The vesicles rupture, scabs are formed, and healing occurs without scarring.

Herpetic infection in man takes two forms regardless of the site of the vesicular lesions. The one is a primary infection, with severe, sometimes fatal, systemic effects, which occurs in individuals who do not show the presence of neutralizing serum antibody. The other, occurring in persons showing

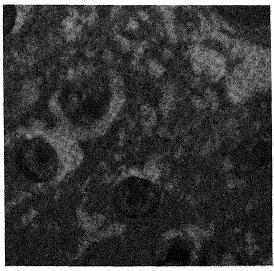


Figure 268. Intranuclear herpetic inclusion bodies in the human brain. (Armed Forces Institute of Pathology, No. 102644.)

neutralizing serum antibody, is characterized by the presence of local lesions but with minor or no systemic effects.

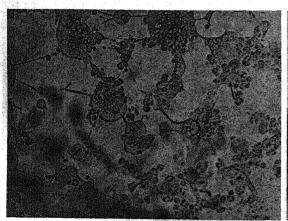
herpes. Primary infections Primary occur relatively early in life. Infants have an effective passive immunity, and infection seldom occurs in the first six months of life. By six years of age the proportion of individuals showing the presence of neutralizing antibody is increasing rapidly, and in adults the proportion of immunes is associated with economic status, 35 to 50 per cent at high levels and 90 per cent or more in lower income groups. The incidence of the primary type of infection is not sufficient to account for the high incidence of neutralizing antibody in adults, and it is probable that the primary infection is often subclinical; Scott has estimated that as much as 80 per cent of primary infections are subclinical.

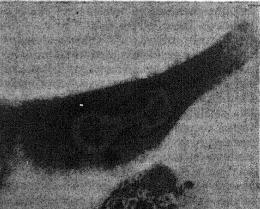
In the nonimmune the vesicular eruption commonly occurs in the mouth to give an acute herpetic gingivostomatitis (aphthous stomatitis, ulcerative stomatitis) in which the vesicular eruption appears as plaques and shallow ulcers; the tonsillar region may be affected, and the gums are often involved. This condition is to be differentiated from herpangina of Coxsackie virus etiology (Chap. Thirty-nine). The submaxillary lymphatics may become swollen and tender, and there is a constitutional reaction of varying severity, including high fever. Occasionally the virus invades the blood stream to give rise to a generalized vesicular eruption known as eczema herpeticum or Kaposi's varicelliform eruption which may simulate mild variola, generalized vaccinia, or severe varicella. The eye may be infected with the production of herpetic keratoconjunctivitis, or the central nervous system involved to give a herpetic meningoencephalitis. There is reason to believe that in the last instance the infection reaches the central nervous system via the peripheral nerves rather than by the hematogenous route. While primary herpes may be fatal, it is usually a self-limited infection, persisting for perhaps two weeks.

Recurrent herpes. Following primary infection, the virus persists in the tissues in spite of the presence of circulating antibody and constitutes an exception to the general rule that a solid immunity to viral infection is associated with the presence of neutralizing antibody. From the point of view of the virus, it is a highly successful parasite, persisting indefinitely in the tissues of the host.⁹⁴

This persistence in the presence of neutralizing antibody is apparently attributable to the spread of infection from cell to cell by direct extrusion. The cytoplasmic membranes of infected cells become altered so that they adhere to adjoining cells with passage of virus across the cell membranes, and in clumps of infected cells the membranes disintegrate to give rise to multinucleated giant cells which are found in the vesicles.¹⁰³

The infection is activated from time to time in various nonspecific ways, often some febrile reaction, so that herpes may





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Figure 269. Herpesvirus in HeLa cell culture. Left, one kind of cytopathology produced by the virus, characterized by the formation of multinucleated giant cells joined by cytoplasmic bridges. \times 100. Right, an infected cell showing division of the nucleus and an inclusion body in each of the daughter nuclei. \times 1000. (Gray: Arch. ges. Virusforsch.)

appear during convalescence from other diseases, physical shock such as chilling, metabolic or hormonal disturbances such as menstruation, etc. Under experimental conditions the infection may be activated by hypersensitivity reactions² or administration of adrenalin;¹⁰⁷ increased secretion of adrenalin occurs in many of the conditions in man associated with activation of herpetic infection. Individuals vary widely in their susceptibility to recurrent infection, *i.e.*, in the severity of the nonspecific stimulus required to induce an attack.

In recurrent herpes the disease is limited to a local vesicular lesion, presumably because of modification of the disease process by the pre-existing immunity. The eruption usually occurs at mucocutaneous junctures such as at the border of the lips or nares (fever blisters, cold sores). It may also occur on the genitalia *(herpes progenitalis) of either sex, on the glans penis or corona in the male and on the labia and lower vaginal mucous membrane in the female.

Chemotherapy. Herpes simplex may be treated effectively with certain halogen substituted uridines, especially 5-iodo-2'deoxyuridine (IDU), which is effective in both man and experimental animals by topical application. 49, 57, 60 The bromo derivative¹¹² and a methylamino derivative⁹⁰ have been found to be effective also under experimental conditions. Another analog, $1-\beta$ -Darabinofuranosylcytosine, has been shown to be effective in the treatment of experimental herpetic keratoconjunctivitis. 125 The discovery of such chemotherapeutic agents is of particular importance in the treatment of herpetic infections of the eye.

Immunity. As indicated above, recovery from primary herpes infection is associated with the appearance of neutralizing antibody in the serum which may be titrated in tissue culture.93, 108 It modifies subsequent recurrent disease but does not prevent it. Complement-fixing antibody appears concurrently, 109 and both antibodies reach peak titer in two to three weeks. The virus, at least that grown on the chorioallantoic membrane, contains two and possibly three kinds of antigens. One of these stimulates the production of neutralizing antibody and is separable by differential centrifugation from the soluble complement-fixing antigen, which also gives a skin reaction on intradermal inoculation into immune individuals. It has been reported that the soluble antigen

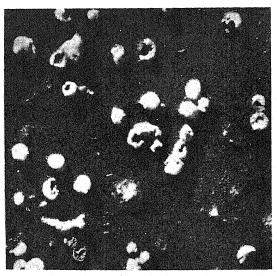


Figure 270. Herpesvirus elementary bodies in an ultrathin section of infected chorioallantoic membrane of the embryonated egg. Some particles are intact, while others show distortion produced by removal of the embedding agent (methacrylate) and the discontinuous structure of the particle. × 25,000. (Morgan: J. Exp. Med.)

is differentiable into a heat-stable and a heatlabile component, the latter giving the skin reaction but the evidence is not altogether consistent. The utility of the skin test has not been established.

Experimental infections. The virus may be grown on various kinds of tissue cultures, including rabbit testis tissue or kidney tissue, where it forms plaques; the occurrence of strains characterized by differences in cytopathic effect (see above) results in the macroplaque associated with syncytium formation, and the microplaque in which the host cells are rounded up. It may also be grown on the chorioallantoic membrane of the 12-day-old embryonated hen's egg, forming pock-like plaques on the membrane which differ from the larger, deeper necrotic pock produced by variola and vaccinia viruses; the typical lesion may not occur until the virus strain has been "egg-adapted" over two or three passages. The common laboratory animals, including the rabbit, mouse, hamster, and rat, may be infected by various routes of inoculation, the results depending somewhat upon the relative dermotropic or neurotropic tendencies of the virus strain. Inoculation of the rabbit cornea results in keratoconjunctivitis and corneal opacity, and this infection, together

established a

with that of the chorioallantoic membrane, is commonly used for diagnostic purposes. Intracerebral inoculation results in encephalitis, which may also occur in the rabbit following corneal inoculation with a neurotropic strain of the virus.

HERPETIC INFECTIONS OF LOWER ANIMALS

Among the variety of viral diseases of lower animals in which a vesicular eruption occurs, at least the three noted earlier are caused by viruses closely similar to the virus of herpes simplex. Although only one of these, B virus, infects man, they may be considered briefly here.

PSEUDORABIES

Pseudorabies (mad itch, infectious bulbar paralysis) is a naturally occurring disease of swine which is relatively mild but highly contagious in this animal. It occurs in Europe and the Americas, and in this country appears, on the basis of neutralizing antibody surveys, to be prevalent among pigs in the Middle West. In the acute form the onset is sudden after a very short incubation period, and the disease is characterized by intense pruritus and late paralysis, with sudden death after one to two days. The disease also affects cattle, horses, goats, sheep, dogs, cats, and other domestic animals, but not man. The common experimental animals, of which the rabbit is one of the most susceptible, are readily infected by any route.

The virus is widely distributed in the body and is present in saliva, but the disease is an acute infection of the central nervous system. It progresses so rapidly that gross signs are not marked other than hyperemia of the meninges and evidence of hemorrhage there and in the lungs. The virus apparently reaches the central nervous system via the peripheral nerves, producing degeneration of the nerve and glial cells in the spinal ganglia and in the corresponding segments of the cord in rabbits. In cattle the nerve and glial cell degeneration occurs in the cerebral cortex and is marked in Ammon's horn.

B VIRUS⁵¹

This virus was originally isolated (1934) from a fatal case of acute ascending myelitis in a laboratory worker who had been bitten by an apparently normal rhesus monkey. It was isolated subsequently directly from the monkey, and there is reason to believe that a benign infection is not uncommon in rhesus monkeys. Human infection is rare, but occurs in laboratory workers and others concerned with handling monkeys.73 The clinical disease is highly fatal, 13 fatalities occurred in 15 cases reported to 1966.95 Rabbits and guinea pigs may be infected experimentally, the virus showing strong neurotropic tendencies, and the virus may be grown on the chorioallantoic membrane of the chick embryo. The virus may be grown in various kinds of tissue cell culture; and variants, characterized by different kinds of cytopathic effects, have been described.34 Neurotropism is less marked in the monkey, and experimental infection produces a generalized infection with a vesicular rash.

The virus is closely similar to that of herpes simplex, being of about the same size and producing intranuclear inclusion bodies in affected cells. It is immunologically related to both herpes simplex virus and pseudorabies virus. Melnick and Banker⁸⁰ found neutralizing antibody in pooled human γ -globulin and in the serum of 11 of 40 persons examined in Bombay, as well as in nine of 44 apparently normal monkeys. It is supposed that the infection is endemic in monkeys, but interpretation of serological evidence, especially in man, must take into consideration the cross-reaction with herpes simplex virus.

VIRUS III

This virus was found in apparently normal rabbits by Rivers and Tillett in 1923 and again by Andrewes and his co-workers.³ The naturally-occurring infection is asymptomatic, but the presence of the virus in infected rabbits is demonstrable by serial intratesticular blind passage to eventually produce lesions in the testicles, skin, and cornea. Intracellular inclusion bodies, closely similar to those of herpes simplex, are found in the affected epithelial and endothelial cells. The virus can be grown in rabbit testis tissue culture, producing

typical intranuclear inclusions. So far as is known, it does not infect man or other animals.

VARICELLA AND ZOSTER

Varicella (chickenpox) and zoster (herpes zoster, shingles) are exanthematous diseases of man characterized by a vesicular eruption and the presence of acidophilic intranuclear inclusion bodies in the affected cells. Zoster, often known as herpes zoster, is quite unrelated to herpes simplex, but both this disease and chickenpox are caused by a virus of the herpes group.

The morphology of the virus particle is closely similar to that of the herpes simplex virus¹ as observed in vesicular fluid, and intranuclear inclusions are produced in cell culture. It is cultured with some difficulty in human cell cultures, amnion, lung fibroblasts with plaque formation, 99 and primary thyroid cell cultures. 15 Serial passage usually requires transfer of infected cells, but heavily infected thyroid cells have been reported to release infective virus into the medium. It has not been possible to infect experimental animals except, perhaps, the monkey.

Varicella. Chickenpox is a common, highly contagious exanthematous disease of childhood which occurs in epidemic form. After an incubation period of two, to

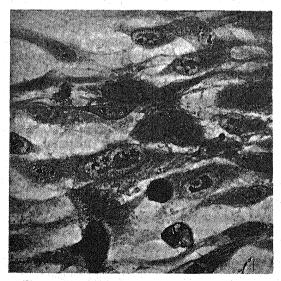


Figure 271. Varicella. Fifth culture passage in foreskin tissue of fluid from varicella lesion. Focus of cells with intranuclear inclusions on sixth day after inoculation. × 550. (Weller: Proc. Soc. Exp. Biol. Med.)

possibly three, weeks, there is a febrile reaction, and a macular eruption appears which progresses to the vesicular stage and may become pustular. These lesions occur in successive crops and may be observed in all stages of development. The prickle-cell layer is affected but without the reticular degeneration which occurs in variola. As the lesion develops, the intercellular edema becomes much more marked than in the variola vesicle, an inflammatory exudate appears, and giant multinucleate cells are present. The corium may form the base of large lesions with consequent scarring, but in general the necrotic changes found in variola lesions do not occur. The lesion is unilocular even at an early stage and can be distinguished from the early multilocular vesicle of variola-vaccinia infections. In the adult there is some tendency toward a varicella pneumonia, which may be less rare than commonly supposed. 68, 84

Some neurotropic tendency of the virus is evident in this disease by the occasional occurrence of encephalitis as a complication, and there may be neuritis of the cranial nerves. The disease is not affected by chemotherapeutic drugs, but chemotherapy is useful in minimizing secondary bacterial infection of the lesions.

Zoster. The vesicular eruption of zoster, or shingles, is substantially identical with that of varicella, but the disease is distinguished from varicella by its sporadic occurrence in adults, inflammation in spinal or extramedullary ganglia accompanied by severe pain localized to some portion of the involved nerve, and the tendency of the vesicular eruption to follow the distribution of one or more cutaneous sensory nerves, usually of the torso.

The disease is initiated by a febrile reaction, general malaise, and extreme tenderness along the dorsal nerve routes. A macular eruption appears within three to four days which becomes vesicular, and pustular when secondary bacterial infection occurs. The dorsal roots of the trunk are most often affected, but involvement of the maxillary or mandibular divisions of the trigeminal nerve may occur, with vesicular lesions in the tonsillar area or buccal mucosa; the ophthalmic division may be affected with scleritis and lesions of the cornea and conjunctiva; or involvement of the geniculate ganglion with corresponding vesicular lesions may occur. Meningoencephalitis is an uncommon complication, and persistent neuralgia tends to occur in older persons. It is clear that the neurotropic tendencies of the virus are much more marked in zoster than in varicella.

Relation of varicella and zoster. It has long been apparent that these diseases are intimately related. While varicella occurs largely in children and zoster in adults, contact with varicella possibly can result in zoster in adults, though convincing evidence is lacking, but zoster seems to have been the source of varicella, at times in epidemic form, in children. The virus particles found in vesicular fluid in the two diseases are morphologically indistinguishable, but evidence of antigenic relationship is inconclusive. Zoster may occur in children or adults who have recovered from varicella, and it is uncertain whether there is a cross-immunity between the two diseases. Some workers have reported substantially complete identity of complement-fixing antigen present in vesicular fluid, but this has not been observed by others. An immunological relation is, however, indicated by the agglutination of varicella virus particles in zoster convalescent serum, though to a lower titer than in homologous convalescent serum, and the fluorescent antibody technique has also shown the presence of common antigens in the two viruses.¹²⁸

It may be concluded, as a working hypothesis, that the two diseases are produced (a) by closely similar viruses, one tending to be dermotropic and the other neurotropic, or (b) by the same virus, some strains of which are sufficiently neurotropic that they are able to persist in the spinal ganglia following recovery from varicella, and the latent infection may be activated by massive doses of varicella virus or other nonspecific insult. 55 It has been suggested also that zoster represents varicella infection modified by a partial pre-existing immunity.

Cat-scratch Fever⁹⁷

Cat-scratch fever (nonbacterial regional lymphadenitis, benign inoculation lymphoreticulosis, cat-bite fever) is a disease of man, apparently acquired from cats, in which there is a primary papular lesion at the site of inoculation which may progress to a small ulcer, and, in a few instances, a transient localized measles-like eruption has occurred. There is a systemic febrile reaction in about two-thirds of the cases and anorexia, malaise, weakness, and aches; occasionally the central nervous system is involved, and there is a regional lymphadenopathy. The immediate regional nodes are inolved first, and subsequently secondary nodes may be affected. There is swelling and tenderness. and the nodes become indurated or suppurate and may require surgical drainage. The affected nodes are encapsulated and occur as three histological types, viz., the acute caseous type or soft tuberculoid granuloma, the acute necrotizing lesion containing a core of polymorphonuclear leucocytes and no definite demarcation from the surrounding tissue, and an epithelioid lesion which is a group of epithelioid cell granulomata with germinal center hyperplasia and no evidence of cellular degeneration.

The epidemiological character of the disease suggests that it is of an infectious etiology. Most cases have a history of skin

injury from a claw scratch or bite of cats, but there is no relation to an obvious illness of the animal. The apparent feline origin of the infectious agent, the nature of the disease in man, and the development of antibody fixing complement in the presence of antigens of the psittacosis-lymphogranuloma group of microorganisms, has suggested that the etiological agent may be a member of that group. But the occurrence of such antibody is irregular, in probably not more than 50 per cent of cases, and this and the failure to react to the Frei test makes such a supposition quite uncertain.⁵

The pus obtained from infected lymph nodes, prepared similarly to the original Frei antigen, has been found to give an apparently specific skin reaction in cases of the disease. 69, 100, 127 This material agglutinates rabbit erythrocytes, and hemagglutination is inhibited by patients' serum and by rabbit antiserum to the pus.²⁷ A hemagglutinating agent has been isolated and passed serially in the allantoic cavity of the embryonated hen's egg. 124 This agent has no cytopathic effect in cell cultures and produces no evidence of infection in the rabbit cornea, but its hemagglutinating activity is inhibited by antiserum to herpes simplex virus. CONTRACTOR OF THE STATE OF

Infectious Mononucleosis⁷¹

Infectious mononucleosis (glandular fever, monocytic angina) is an acute, presumably infectious, disease occurring in children and young adults characterized by an increased number of monocytes and large lymphocytes in the blood, angina, lymphadenitis, and the presence of heterophile antibody in the serum. It is known in Japan by a variety of names, including Tokushima fever and Kagami fever. A similar disease of lower animals in which the central nervous system may be involved with some frequency is caused by the bacterium Listeria (Chap. Twenty-seven), monocytogenes which also occurs as a natural infection of man. This microorganism is not found in the great majority of cases of human disease with the syndrome characterizing infectious mononucleosis. While there is no definitive supporting evidence, it is generally assumed as a working hypothesis that this disease is of viral etiology.

The disease in man.33 The onset of the disease may be acute or insidious and is characterized by loss of appetite and fatigue. Systemic involvement is indicated by the febrile reaction and symptoms such as epistaxis, nausea and vomiting, and headache; the cervical lymph nodes become swollen and tender, and there is angina ranging in severity from mild hyperemia to a pseudomembranous ulcerating form. A cutaneous rash occurs in some individuals, there may be conjunctivitis and/or evidence of liver damage, and the central nervous system is occasionally involved. The white blood cell count increases, to as much as 80,000, the differential count showing a decrease of polymorphonuclear leucocytes and a marked increase in monocytes to give a characteristic blood picture.

The serological evidence of infection is an

increased titer of heterophile antibody, demonstrable by agglutination of sheep erythrocytes (the Paul-Bunnell test) over the normal titer of 1:50 to 1:60; a titer of 1:80 is generally regarded as the minimum which may be taken as diagnostic. The hemagglutination test may be made more specific by a preliminary absorption of the serum with boiled guinea pig kidney tissue, which removes "nonspecific" antibody; the antibody associated with this disease is absorbed by treatment of the serum with boiled sheep erythrocytes. The goat erythrocyte is reported to give a more sensitive test, and a number of variations, including an ox erythrocyte hemolysis test and the use of trypsinized bovine red cells have been suggested.21

The disease is thought to be transmitted by intimate contact such as kissing, and it has in fact been called "kissing disease." It is not ordinarily possible to trace contact between infected persons; this has been interpreted by some to be indicative of a low degree of contagiousness and by others to suggest that subclinical infection is very common.

Experimental infections. Transmission of the infection, evidenced by mild lymphadenitis and small increases in the monocyte count, to the monkey has been reported by some workers but not by others. It has not been possible to cultivate an etiological agent in the embryonated egg, and human volunteer experiments have given equivocal results. More recently Japanese workers have produced a disease resembling mononucleosis in monkeys by parenteral, but not oral, inoculation with *Rickettsia sennetsu*, 111 an organism isolated in 1954 from persons ill with disease diagnosed on clinical grounds as infectious mononucleosis.

The Cytomegaloviruses¹¹⁶ (Salivary Gland Disease Viruses)

A disease known as salivary gland disease occurs widely in man¹¹⁷ and in a number of animal species examined, including rats, mice, guinea pigs, hamsters, Cebus monkeys, and chimpanzees. It is characterized by cellular gigantism and intranuclear inclusion bodies (and sometimes small compact intracytoplasmic inclusion bodies) present within the affected cells, whose size and

structure tend to set them off from the inclusion bodies observed in other viral diseases.⁷⁴ This disease, also known as cytomegalic inclusion disease, appears to be caused by a group of closely related, but highly species-specific, viruses.

The naturally occurring infection in lower animals seems to be almost invariably asymptomatic. It is probably usually sub-

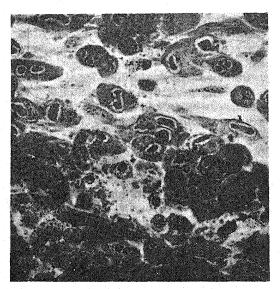


Figure 272. Salivary gland virus in human foreskin tissue culture showing the edge of a focal lesion, with central degeneration and infected cells at the periphery. Hematoxylin and eosin; × 360. (Weller: Proc. Soc. Exp. Biol. Med.)

clinical in man also, but severe disease occurs in infants,46 commonly in those under two years of age, and about 100 fatal cases have been reported in all; in older children it may be secondary to some other disease. The infection is generalized, and the typical histopathology is found at autopsy in the salivary, most often submaxillary, glands, in the kidneys, liver, lungs, pancreas, and occasionally in the brain. The virus is found in the saliva and urine. The infection persists for extended periods as indicated by isolation of virus from the urine for as long as four years, 129 and it is believed by some that, like herpes simplex virus, it may persist indefinitely.

The causative viruses have been isolated in salivary gland disease of the mouse, guinea pig, and man, in the last from salivary glands, kidneys, and adenoids, in tissue culture of homologous fibroblasts of varied tissue origin. 104, 115 After not less than 30, and up to 70, days of incubation, focal areas of degeneration appear on the fibroblast sheet which are separated by normal cells and which may become confluent on continued incubation. The cells in the affected areas contain the typical large acidophilic intranuclear inclusion bodies. While such cultures may be maintained in serial transplant, the virus grows only on homologous fibroblasts - i.e., mouse virus will not grow on human fibroblasts, or human virus on mouse fibroblasts - and cannot be grown on the usual HeLa cell, monkey kidney, or rabbit trachea tissue cultures. Active virus is present in the homologous fibroblast cultures: intraperitoneal inoculation young mice with cultured mouse virus produces a fatal disease characterized by visceral necrosis; and a fatal infection is produced by the intracerebral inoculation of fetal or very young guinea pigs with homologous culture virus, but human virus will not infect these animals.

The cytopathic effect in fibroblast culture is inhibited in the presence of antiserum, and antigen fixing complement in the presence of antiserum is present in tissue cultures. By such tests the human strains of the virus are shown to be immunologically distinct from varicella and measles viruses and show a degree of serological heterogeneity among themselves. They are morphologically indistinguishable from the other herpes viruses. 114, 134

The general occurrence of the disease in man is indicated by the incidence of typical histopathology in 10 to 30 per cent of infants at autopsy regardless of the immediate cause of death, the isolation of the virus from apparently normal adenoidal tissue, and the distribution of complement-fixing antibody. The observed incidence of significant titers of this antibody has been 14 per cent in the six- to 23 months age group, 53 per cent in the 18- to 25-year age group, and 81 per cent in persons over 35.133

Tumor Viruses 25, 44, 75, 78, 121

The property of some viruses of inducing proliferation of the affected cells has been alluded to earlier. In many instances proliferation is followed by necrosis, relatively rapidly in a disease such as smallpox in which the proliferative stage is so transitory

as to be almost inapparent, and more slowly in the epitheliomatous type of fowlpox infection. In infections with other viruses morphologically closely similar to the poxviruses, molluscum contagiosum in man and myxoma and fibroma in rabbits, the proliferative response predominates, but necrosis occurs eventually.

Still other viruses, notably that of rabbit papillomatosis, enter into a continuing, benign relationship with the host cell, and an orderly proliferation may continue for extended periods, but eventually the growth becomes disorderly, invades contiguous tissues, metastases occur, and the rapidly proliferating cells develop as a malignant process; i.e., the proliferative process has become cancerous. The mammary carcinoma of the mouse associated with the virus-like milk factor is similar in that the activity present in the milk produces no immediately apparent effect but is essential to the subsequent development of the carcinoma. In still other instances, such as avian sarcoma and leucosis, the proliferative response is malignant substantially from the initiation of the viral infection. Thus a continuous series of types of cellular proliferative response to viral infection may be constructed.

The relation of the virus to unrestricted proliferation is not clear, for evidence of its indefinite persistence in the affected tissues may be equivocal or lacking. In some instances the virus may not be directly separable from the affected tissues, but its continued presence and increase in amount are inferred from rising antibody titer to it in the blood, the infection persisting in masked form presumably because of the concurrent presence of antibody. In the case of avian leucosis virus prepared and studied in highly purified form by Beard, Sharp, and their colleagues, the viral agent, although extrinsic to the host cell and not arising de novo from it, nevertheless contains host tissue antigen as an integral portion of the viral substance, and the differentiation of virus and host cell tends to blur. In still other instances, the virus is detectable only in the earlier stages of the neoplastic process. and thereafter there is no evidence of its presence; under such circumstances the virus would appear to function, in current terminology, as a carcinogen, possibly by incorporation into the genome of the host cell.64

As yet it has not been possible to associate viruses with cancerous disease in man, 87, 101, 123 but a number of viruses are oncogenic in warm-blooded animals. These may be considered to fall into three groups:

(1) Poxviruses, including molluscum contagiosum and the fibroma-myxoma viruses

(2) Papovaviruses (papilloma-polyoma-vacuolating viruses) and some types (7, 12, 18, 31) of the adenoviruses (Chap. Thirty-eight)

(3) The avian leukosis, mouse leukemia, and mouse mammary cancer viruses. Of these, viruses of the first two groups are DNA viruses, and those of group 2 are 40 to 80 m μ in diameter, are ether-sensitive, have cubic symmetry, and are not enclosed in an envelope or limiting membrane. Those of the third group are RNA viruses approximately 100 m μ in diameter, of uncertain, possibly helical, symmetry, enclosed in an envelope, and ether-resistant. Clearly, the oncogenic viruses are heterogeneous by the usual criteria.

MOLLUSCUM CONTAGIOSUM

Molluscum contagiosum is an infectious disease of man, world-wide in distribution and occurring in both sporadic and epidemic form. The infection is spread by contact, and the disease is characterized by the occurrence of multiple nodules on the face, arms, back, and buttocks which are at first red, then pearly white, and undergo necrosis with the discharge of caseous material. The disease is chronic, and the lesions persist over several months.

The lesion develops in the epidermis as thickened areas of hyperplasia and hypertrophy of the affected cells. There is little or no reaction in the corium in the absence of secondary bacterial infection, and the pathological changes become more marked proceeding from the germinal layer to the surface of the epidermis. The affected cells are enlarged and practically filled with an acidophilic granular inclusion body, molluscum body, similar to those observed in the pox diseases.6, 17 The inclusion body contains oval or brick-shaped elementary bodies 230 by 330 m μ in size. In ultrathin sections the elementary bodies are heterogeneous with respect to contained electrondense material, and the several morphological types may be interpreted as representing a sequence of developmental stages as described elsewhere41,79 (Chap.

It has not been possible to infect experimental animals with this virus nor to grow it on the chorioallantoic membrane of the embryonated egg. Heat-labile soluble antigen may be prepared as extracts of macer-

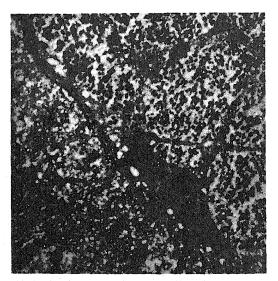


Figure 273. Electron micrograph of cell infected with molluscum contagiosum virus. Strands of cytoplasmic material separate the pockets of mature virus, and the nucleus is pushed to the edge of the cell. × 8500. (Gaylord and Melnick: J. Exp. Med.)

ated infected tissue, which fixes complement in the presence of serum of some patients and has been shown to be unrelated to the antigens of the poxviruses.

MYXOMA AND FIBROMA VIRUSES

Infectious myxomatosis of rabbits is a naturally occurring mild disease of the South American rabbit (Sylvilagus braziliensis) and a rapidly fatal disease of the European wild rabbit (Oryctolagus cuniculus) and the common laboratory rabbit derived from it. The disease was described by Sanarelli in 1898 as occurring in laboratory rabbits in Montevideo. Infectious fibromatosis is a naturally occurring benign disease of wild cottontail (Sylvilagus species) rabbits, described by Shope in 1932, characterized by fibromata which eventually regress. The viruses causing these diseases are closely related immunologically, are morphologically indistinguishable, and are transmitted by arthropods, and the fibroma virus may be transformed to myxoma virus.

Infectious myxomatosis. While the virus of myxoma produces only benign tumor-like masses in Sylvilagus rabbits, the infection in Oryctolagus rabbits produces a general-

ized disease that is almost uniformly fatal in one or two weeks. The naturally occurring infection is transmitted mechanically by biting arthropods, including mosquitoes and rabbit fleas, and artificial infection may be produced by pricking the skin with a contaminated needle.

After multiplication at the site of inoculation, the virus spreads through the lymphatics and into the blood steam and is distributed throughout the body. Both epithelial and connective tissues are affected, with destruction of epithelial cells and proliferation of connective tissue. The lesions in the skin, both primary at the site of inocuation and secondary in the skin, especially about the face and anogenital region, in the lymph nodes, and in the spleen, are tumorous growths. These are characterized by varying degrees of inflammation, the deposition of a glairy mucinous fluid that appears in stained sections as a fibrinous exudate, and the presence of characteristic stellate cells sometimes called myxoma cells.

The myxoma cells are enlarged macrophage-like cells with a heavily staining nucleus, and they contain the virus, sometimes as dispersed elementary bodies in the cytoplasm, but more commonly in intracytoplasmic inclusion bodies closely similar to those found in the pox diseases. 40 The elementary bodies are oval or brick-shaped particles, of the same order of size as those of the pox diseases and molluscum contagiosum.

The virus may be grown on the chorioallantoic membrane of the embyronated hen's egg, where it produces pock-like plaques, and it grows in rabbit kidney tissue culture, ¹⁶ producing inclusion bodies in typical stellate cells. A heat-labile soluble complement-fixing and precipitating antigen is formed in culture and is present in infected tissues and also in the serum of infected animals, but antibody to this antigen is not identical with protective antibody. The virus is closely related immunologically to the fibroma virus (see below).

Myxomatosis has been of considerable epidemiological interest following its deliberate introduction, in 1950 and 1951, into Australia for biological control of the native rabbit population. The unique opportunity to study the natural history of an infectious disease has been thoroughly exploited by the Australian workers.³⁹ The

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disease was also introduced in France in 1952 and spread rapidly throughout Europe.

fibromatosis. This which is transmissible to laboratory (Oryctolagus) rabbits, is similar to infectious myxomatosis, but it is not fatal. The characteristic lesion is a benign subcutaneous spindle-cell fibroma in which there is infiltration of inflammatory cells and hyperplastic and degenerative changes in the overlying epidermis reminiscent of the lesions of molluscum contagiosum in man. Closely similar intracytoplasmic inclusion bodies occur in the affected cells, and also in infected cells in rabbit testis tissue culture, but the virus does not grow in cultures of human foreskin or monkey kidney tissues.62 The contained elementary bodies are closely similar to those of the myxoma virus and poxviruses.

The fibroma virus is immunologically closely related to, but not identical with, the myxoma virus. Animals recovered from fibromatosis are partially immune to myxoma in that they do not contract the disease, but they may be infected and become healthy carriers of myxoma virus. The few rabbits which recover from myxomatosis, however, appear to be solidly immune to fibromatosis.

Fibroma-myxoma transduction. 63 The intimate relation between the viruses of fibroma and of myxoma is indicated by the transformation of the former to the latter by a process which is apparently analogous to the in vivo transduction of pneumococcus types, etc. (Chap. Seven). This was first shown in the rabbit by the simultaneous inoculation of heat-inactivated myxoma virus and living fibroma virus in order to give myxomatosis in the rabbit. This transduction has also been carried out in rabbit testis tissue culture. The transforming agent prepared from myxoma virus is sedimentable by high speed centrifugation and associated with a particle similar in size to that of myxoma virus. It has been suggested that the transforming principle, which is resistant to heat and ether treatment, consists of intact DNA contained within a membrane of denatured protein.

The result is necessarily an alteration in the directed synthesis of viral substance by the host cell initiated by the presence of the inactive virus, and it is of special interest that this can occur in the more complex system involving the host-parasite relationship as well as within the self-contained synthetic mechanisms of bacteria.

INFECTIOUS WART VIRUS

The virus of human warts is a papovavirus closely related to the papilloma viruses rather than to the molluscum contagiosum poxvirus. It occurs in the nuclei of infected cells as crystalline masses. The virus particles have cubic icosahedral symmetry, and are 45 m μ in diameter. The virus has been grown in cell cultures83,92 and found to be infectious for man. Previously the disease had been transmitted from man to man with the production of warts after an incubation period of some months. The lesion, essentially a proliferation of the Malpighian layer of the skin, is usually benign and tends to regress in time, but genital warts may become malignant.

RABBIT PAPILLOMA VIRUS¹²²

Infectious rabbit papillomatosis, described by Shope and Hurst in 1933, is transmissible by cell-free filtrates and is of viral etiology. The virus has been prepared and studied in highly purified form²⁵ and found to be a spherical particle, 40 to 50 m μ in diameter, morphologically indistinguishable from the viruses found in human warts⁸¹ (see above). It resembles other viruses in chemical composition, containing 8.7 per cent nucleic acid of the deoxypentose type, but less lipid, 1.5 per cent, than many other viruses.

Infectious papillomatosis. The disease in cottontail rabbits is characterized by warty growths on various parts of the body. It may be transmitted serially in these animals but not all are susceptible, and in half or more of those infected there is eventual regression of the papillomata; malignant change is rare. The domestic rabbit is much more susceptible, for papillomatous disease develops readily; the tumors grow in benign form for some months and then become malignant, killing the animals. The disease in the domestic rabbit usually cannot be transmitted by cell-free filtrates in serial passage, and the presence of the virus in the affected tissue is indicated by the antibody response.

Pathogenesis of papillomatosis. The pathogenesis of this disease has been one



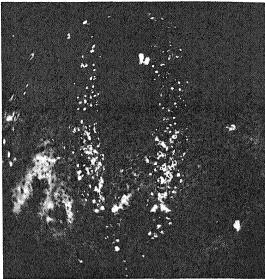


Figure 274. Rabbit papilloma. Left, a paraffin section stained with hematoxylin and eosin and showing the basal, granular intermediate and the superficial keratinized epithelial layers of the papilloma. Right, a frozen section treated with fluorescent antibody for the Shope viral antigens; the localization of the antibody in the nuclei of the keratinized epithelial cells of the papilloma is shown by the bright areas. × 100. (Mellors.)

of the most thoroughly studied of diseases of this kind, and in at least some respects may be regarded as a prototype. The histopathology has been summarized by Kidd.61 On inoculation of the scarified skin the virus induces a prompt and marked proliferation of the epidermal cells, those of the stratum spinosum showing perinuclear vacuolation and dyskeratosis, but the cells mature to a point. They do not form the characteristic horny layer, but rather an atypical cornification occurs in which each cell is surrounded by a layer of keratin and fuses with adjacent cells to form a honeycomb structure, and the cell contents disappear. With extension at the base, the developing cells are pushed into folds and papillae, and keratinization without desquamation results in the horny mass rising above the level of the skin and constituting the benign papilloma. Viral antigen is demonstrable in the nuclei of the keratinized epithelial cells by the fluorescent antibody technique as illustrated in the accompanying figure.

The benign papillomata develop and persist in this form for some months, but eventually there is an abrupt change in the growth, occurring within a relatively short time in all of the lesions on a given animal. Histologically, the germinal cells, which to this point are arranged in an orderly

syncytium, assume a disorderly arrangement, are devoid of perinuclear vacuoles and keratohyalin granules, and do not undergo the abnormal cornification described above. They displace the cells of the basal and spinous layers and undermine the horny mass, which sloughs off. This new type of cell then breaks through the basement membrane, invading contiguous tissues, metastasizes, and assumes the character of a squamous cell carcinoma. Until this last stage, serological evidence indicates that the virus persists in the affected tissue. both in the original animal and in recipients of tissue transplants, but eventually the virus seems to disappear entirely.

Factors affecting malignancy. While it seems clear that at least the initial proliferative response which gives rise to the benign tumor is induced by the virus, there is no evidence that the virus plays a significant part in the subsequent change to malignancy, and there is some reason to believe that other factors may be involved. For example, the benign papilloma can be made rapidly cancerous by the application of carcinogens such as methylcholanthrene and tar-induced epithelial tumors to bring about an abrupt change to malignancy; in the latter instance the virus would appear to behave as a carcinogen.

VACUOLATING VIRUS^{56, 119}

This papovavirus, also known as SV40 (simian virus 40), is commonly found in rhesus and cynomolgus monkey kidney tissue, and was originally discovered as a contaminant of kidney cell cultures and of living virus vaccines, notably the attenuated poliovirus vaccines, prepared in such cultures. It is readily cultivable in grivet, vervet, and patas monkey kidney cell cultures, and in rhesus testicle cultures, to give a unique cytopathic effect characterized by ballooning and vacuolation of the infected cells. In newborn hamster kidney tissue culture it induces malignancy as evidenced by marked growth stimulation.98 It also produces tumors on inoculation into neonatal hamsters.31,43 It is immunologically unrelated to the polyoma virus.

VIRAL AGENTS OF MOUSE NEOPLASTIC DISEASE

The mouse suffers from a variety of neoplastic diseases, susceptibility to which often has a genetic background, and has been an extremely useful experimental animal.

Polyoma.^{30, 47, 118} Various kinds of tumors may be produced in mice by the inoculation of cell-free extracts or filtrates of mouse tumors, including leukemic tissue, shortly after birth. For the most part multiple effects are produced; *i.e.*, it is ordinarily not possible to predict the location or character of the neoplastic growth so produced.

A possible viral nature of the activity present in such cell-free preparations is suggested by its small size and separation from intact tumor cells. The activity demonstrable in cell-free preparations of several mouse tumors has been found to be cultivable in mouse embryo tissue culture, producing a cytopathology characterized by the appearance of small patches of pyknotic cells which progresses until the majority of the cells are affected in two weeks. The activity increases from 10^3 TID₅₀ at two weeks to as much as 106 TID₅₀ at three weeks, and the infectivity for intact experimental animals is correlated with the cytopathic effects and is neutralized by antiserum. The viral agents so cultivated are nonspecific with respect to the type of neoplastic growth produced in the intact animal and have been designated polyoma virus. The virus is spherical, 30 to 40 m μ in diameter, and resembles the other papovaviruses in its properties.

A total of 23 kinds of different primary neoplastic lesions, including pleomorphic tumors of mucous glands, adenosarcomas, renal and bone sarcomas, and epidermoid carcinomas, are produced. It is of interest also, and in contrast with the genetic element in the occurrence of mammary adenosarcoma in the mouse, that such growths are produced regardless of strain of mouse and can be produced also in hamsters.

Animals can be passively immunized to the virus, providing that antiserum is given prior to, or admixed with, the inoculum, but not when serum is given as early as one hour after inoculation. The infection appears to be common in mice in latent form, perhaps because of immunity, initially of maternal origin. Viral activity is present in the neoplasm, but when the tumor is passaged by transplantation viral activity may disappear in the course of time, perhaps after 10 transplants, even though the tumor remains transplantable. The nature of refractoriness to transplantable tumor is not dependent upon antibody and is of particular interest.

Leukemia. 45, 86, 102, 113 Although Rous described cell-free transmission of chicken sarcoma many years ago, interest in this aspect of neoplastic disease remained essentially dormant until Gross demonstrated the viral etiology of a mouse leukemia in 1951 to initiate a period of intense interest and activity. Since then a number of leukemogenic viruses, active in various inbred strains of mice, have been described. The majority produce lymphocytic neoplastic disease, but some produce reticular cell neoplasms and myeloid neoplasms. These strains have been derived from spontaneous leukemias and other neoplastic disease, and from such disease occurring in irradiated animals. They differ somewhat in particle size, over a range of 70 to 100 m μ , and are immunologically distinct but show some interrelationships. They are commonly identified by the name of the investigator(s) who originally described them; the first is the Gross strain, and the others are the Kaplan, Schoolman and Schwartz, Moloney, Friend, etc., strains. The origins and properties of these strains are summarized in the accompanying table.

Murine Leukemia Viruses*

STRAIN (INVESTIGATOR)	ORIGIN	HOST SUSCEPTIBILITY				
		AGE			PARTICLE	NEOPLASTIC
		suckling %	ADULT	STRAIN	SIZE (mμ)	CELL TYPE
Gross						
Passage A	AK, C ₅ 8 (spontaneous leukemia)	62-100	44–60	C ₃ H, C ₃ Hf (Bitt- ner) (rats)	89	Lymphoid
Passage X	C ₃ H/Gs (x-irradiated)	65		C ₃ H		Lymphoid
Schoolman and Schwartz	Swiss, C ₃ HeB (x-irradiated)		15–100	Swiss, C ₃ H	102	Lymphoid sarcoma
Kaplan	C ₅₇ BL/Ka (x-irradiated)	69		C ₅₇ BL/Ka,F ₁ hybrids		Lymphoid
Breyere and Moloney	Plasma cell neoplasm (70429)	100	100	BALB/c,C ₃ H, (rats)	100	Lymphoid
Moloney	Sarcoma 37	100	100	BALB/c,C ₃ H, C ₃ Hf, I, C ₅₇ _ BL,DBA/2, RIII Swiss	98	Lymphoid
Friend	Ehrlich sarcoma		85–100	Swiss, DBA/2	90	Reticulum – erythro- blastosis
Stansly	Ehrlich sarcoma	92-100		BALB/c		Reticulum cell sar-
						coma B
Graffi	Sarcoma I, Sarcoma II, SOV 16, Ehrlich carcinoma, Sar- coma 37		70	Agnes Bluhm, sg, db, (rats)	70	Myeloid

^{*}Modified from Moloney.86

Mammary carcinoma.^{22, 88} Three factors are involved in the development of mammary adenocarcinoma of the mouse, viz., the genetic constitution of the animal, estrogenic stimulus, and a filterable activity present in cell-free preparations or milk factor. While the milk factor persists and increases in amount in the tissues of the host, hyperplasia of mammary acini occurs only in conjunction with estrogenic stimulation. Some of these become malignant adenocarcinomas, but the relation of the milk factor to malignancy is not clear.

Attempts to characterize the activity have been only partially successful. It is widely distributed in the tissues of mice harboring it, and various tissue extracts are as effective as milk. It obviously increases in amount in that it is transmitted serially in mice under natural conditions. It passes the usual bacteria-proof filters such as the Seitz and Berkefeld. It is highly active, in dilutions as high as 10⁻⁶ in breast tissue extracts. The activity is resistant to tryptic digestion and is destroyed by heating tissue extracts to 60° C. for one hour. It is sedimented at 23,000 × G

in 90 minutes, and some workers have reported that the activity is associated with spherical particles about 30 m μ in diameter, but there is no unequivocal evidence that these represent virus particles. Cell-free activity does not replicate in the embryonated egg but may be recovered from tumor tissue carried for as many as eleven passages in the chick embryo. Attempts to characterize the activity chemically and immunologically have given essentially negative results.

VIRUSES OF AVIAN NEOPLASTIC DISEASE¹⁰⁶, ¹²⁶

The viral etiology of what is now known as the avian leucosis complex was described by Ellermann and Bang in 1908 and the fowl sarcomas transmissible by cell-free filtrates by Rous in 1911. The former is an infectious disease characterized by an autonomous proliferation of cells of hematopoietic tissues. The Rous sarcomas have

been thought to originate in monocytes or histiocytes, but there is evidence that the host cell of the causative virus is the fibroblast. In contrast with rabbit papillomatosis, malignancy is appararent at the initiation of these diseases.

Avian leucosis. 10 Avian leucosis is not a single disease, but a complex of a variety of forms of disease. Beard, Sharp, and Eckert¹¹ divide this complex into two categories, the one lymphomatosis and the other erythromyeloblastic leucosis. In the former, cells of lymphoid origin are involved, the affected cells occur in fixed tissues rather than in the circulation, and the disease process varies with respect to the localization of the primitive cells affected, viz., neurolymphomatosis, visceral lymphomatosis, isolated lymphosarcomatous tumors, etc. The second group is made up of erythroblastosis and myeloblastosis, or a combination of both as erythromyeloblastosis, and is characterized by the occurrence of very large numbers of primitive cells in the circulating blood.

While these diseases "breed true" in that cell-free filtrates of lymphomatosis produce lymphomatosis, and erythromyeloblastosis is similarly reproduced, cellular transfer of the former has resulted in the latter disease. It is quite uncertain whether the several differentiable conditions are produced by a single virus, by variants of a single virus, or by a group of closely related viruses.

Erythromyeloblastosis. While a particulate component has been isolated in studies on lymphomatosis, the most detailed studies have been carried out with the virus of erythromyeloblastosis, and it may be considered here as representative.

There is a great technical advantage in the study of this virus, for it is present in the plasma of infected chickens in such large amounts that the plasma is literally turbid, and it may be sedimented by high-speed centrifugation. The virus particle is spheroid in shape, with an average diameter of about 120 m μ , and is apparently a relatively fragile structure, for it is pleomorphic in dried preparations, like the virus of Newcastle disease.26 Its chemical constitution is not known with precision, other than its relatively high content of lipid, as much as 30 per cent, in contrast to rabbit papilloma virus. It is of special interest that it contains adenosine triphosphatase activity, which appears to be an integral part of the virus particle.

The serum of infected chickens does not

ordinarily contain antibody to the virus in regularly demonstrable amounts, but it functions as an antigen in concentrated preparations. Three kinds of antigens are found, one also present in normal chicken tissue, heterophile or Forssman antigen, and virus-specific antigen. The occurrence of host-specific antigen in purified preparations of viruses is commonly assumed to be, and probably often represents, contamination. Such antigen present in influenza virus is suspected to be an integral part of the virus particle, but in the case of this leucosis viral antibody to the chick tissue antigen and that to Forssman antigen, as well as antibody to the virus-specific antigen, neutralize the viral activity, which appears to establish all three kinds of antigens as structural components of the virus.

Fowl sarcoma.^{50, 105} Rous and his associates were able to transmit several types of chicken sarcomata by means of cell-free filtrates to produce in the recipients under appropriate experimental conditions the same kind of tumor. That designated tumor 1, which has been used extensively for experimental purposes, was a spindle-cell sarcoma characterized by free metastasis and the production of disease fatal in about a month. Tumor 7 was an osteochondrosarcoma, and tumor 18 a spindle-cell sarcoma characterized by the presence of blood sinuses in which growth occurred.

It was early observed that, after several transplants, variations began to occur in tumor 1 as indicated by the appearance of spherical rather than spindle cells and hemorrhagic growths, changes possibly attributable to variations in host resistance as well as in the tumor. Other variations may be produced experimentally. When inoculated in very large amounts in ducks this virus produces two kinds of tumors. The one appearing only after several months is no longer effective in adult chickens and produces a generalized disease characterized by periosteal sarcomata in the duck, while the other which appears much earlier is not found to be modified on subsequent infection of the chicken. The variation apparent in the first instance can be reversed by inoculation of the variant into young chicks to produce again the two types of lesions found in ducks, one of which represents the reversion. Other variants produced in ducks have been found to have altered tissue specificities in chickens, and still other

variants have been produced in turkeys and guinea fowl.

The virus of tumor 1 has been prepared in purified form by both chemical and physical methods, beginning with its salting out by half-saturated ammonium sulfate in association with a nucleoprotein fraction. There appears to be some variation in the size of the infective particle, and it is usually given as within the range 70 to 100 m μ . Particles of this size have been reported to occur in the affected tissue, for example as round or oval bodies within the walls of vacuoles of the cells, showing a double limiting membrane surrounding a central electron-dense body,32 but in general it has not been established that such bodies are virus particles. Purified preparations of the virus give about 8 per cent total nitrogen, 35 per cent lipid, and 1 to 2 per cent pentose nucleic acid. The virus has been grown in tissue culture.⁷⁶ high dilutions of inoculum producing, in chick embryo tissue culture, discrete foci of cytopathology in the monolayer, characterized by a swelling and granularity of the cells and eventual displacement of the nuclei, proportional in number to the infectivity of the inoculum.

The antigenicity of purified virus preparations is separable into two components, the one having virus specificity and the other that of normal chicken tissue; whether the latter represents an integral part of the virus or contamination is not clear. It is of particular interest that some of the tar-induced tumors are immunologically related to the Rous sarcoma 1 virus; for example, pheasants inoculated with a nonfiltrable tar-induced sarcoma produced antibody to the Rous virus. It has been reported also that certain carcinogen-induced tumors are subsequently transmissible by cell-free preparations.

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THE MYXOVIRUS GROUP (INFLUENZA, MUMPS, MEASLES, RUBELLA, RABIES) AND RELATED VIRUSES

Disease of the respiratory tract in man is of heterogeneous etiology, the causative agents ranging from bacteria and Mycoplasma, through fungi, rickettsiae, and psittacosis-ornithosis organisms, to various kinds of viruses. Acute respiratory disease, often referred to as ARD, is thus etiologically complex, though perhaps less so clinically.

Elucidation of the viral etiology of a portion of diseases of this kind was initiated with the isolation and characterization of influenza virus in 1933 through reproduction of the disease in the ferret. Subsequent development of more sophisticated tissue culture techniques, coupled with the availability of antibiotics to minimize the effects of bacterial contamination of specimens such as nasopharyngeal washings, while not affecting the proliferation of the virus, made possible wide application of this method and the isolation of a series of hitherto unknown viruses. 124 The extension

of readily applicable serological methods of characterization and identification as a corollary to tissue culture methods, and the application of such serological methods to the retrospective diagnosis of infections of this kind, allow estimation of the relative prevalence of such viruses in the host population. In consequence, a general pattern of viral infections of this kind is beginning to emerge, 25, 103, 125 although many gaps remain and a considerable portion of undifferentiated acute respiratory disease, or ARD, remains of as yet unknown etiology.

The viral agents of ARD are diverse, including not only the influenza and related viruses of the myxovirus group, but also adenoviruses, and certain of the enteric viruses (picornaviruses) associated with the common cold syndrome. The myxoviruses and adenoviruses will be considered here, and the picornaviruses, including those producing respiratory disease, elsewhere (Chap. Thirty-nine).

The Myxoviruses

The viruses of the myxovirus group, socalled because of the affinity of some for mucinous substrates (see below), are ether sensitive DNA viruses in which the nucleic acid is considered to be single-stranded; the capsid has helical symmetry with a helix diameter of 9 to 17 m μ ; and the mature virus particle, assembled at the host cell surface but completed outside the cell, is enclosed in an envelope.

This group of viruses tends to separate into two subgroups, designated subgroup I and subgroup II, or myxovirus and paramyxovirus, on the basis of size and cytopathic effect (CPE) produced in cell cultures. The first group is somewhat more uniform and smaller in size, 80 to 120 m μ versus 150 to 250 m μ . The myxovirus CPE in cell culture tends to be degenerative in type, while that produced by the paramyxoviruses is syncytial in character and eosinophilic inclusions are found in the cytoplasm of the affected cells. The myxovirus subgroup includes the influenza viruses and probably the rabies virus, and the paramyxovirus subgroup the parainfluenza, mumps, measles, and Newcastle disease viruses.

Hemagglutination. These viruses act as hemagglutinins as do the arboviruses (Chap. Forty), but are distinguished from the latter by their content of neuraminidase, which degrades the receptor substance on the red cell to allow spontaneous elution. Some other viruses, such as certain of the poxviruses, produce a soluble hemagglutinin but the virus particle does not act as an agglutinating agent.

Hemagglutination by myxoviruses provides a convenient method of in vitro titration of the virus; i.e., serial dilutions of virus-containing material, such as allantoic fluid, in red cell suspensions show an end point that is a measure of the viral concentration, which has been particularly useful with the influenza viruses (see below). Agglutination of red cells also occurs about centers of viral proliferation in cell culture sheets, i.e., plaques, and occurs prior to, or in the apparent absence of, CPE; the phenomenon is termed hemadsorption. Viral hemagglutination is specifically inhibited by antiviral serum, and the hemagglutination-inhibition (HI) test may be used to titrate antiviral antibody (see below).

When the virus-agglutinated red cells are allowed to stand for a few hours, the virus

particles are spontaneously eluted in consequence of degradation of the red cell receptor substance, and are no longer agglutinable by the addition of fresh virus. Neuraminidase is also produced by a number of bacteria, notably *Vibrio cholerae* and *Cl. welchii*, and treatment of red cells with cell-free supernatants of liquid cultures of such bacteria renders them inagglutinable by myxoviruses; neuraminidase of such bacterial origin is commonly called receptor-destroying enzyme, or RDE.

The cell receptor substance is a mucoprotein, protein conjugated with oligosaccharides. The size of the oligosaccharide groups and the number of them attached to the protein vary. The terminal units of the oligosaccharides are acetylated neuraminic acid residues, attached by a glycosidic linkage to the adjacent sugar. It is this linkage which is destroyed by neuraminidase, or RDE, with consequent breakdown of the orderly arrangement of groups apparently necessary for the adsorption of virus.

The myxoviruses are not identical in that, while red cells treated with one virus are no longer agglutinated by, or adsorb, that virus, they may or may not adsorb other viruses of the group and be agglutinated by them. This has led to the formulation of a receptor gradient series: mumps → NDV \rightarrow influenza A \rightarrow swine influenza \rightarrow influenza B. Red cells treated with mumps virus will still agglutinate in the presence of NDV and the influenza viruses; those treated with NDV will not be agglutinated by mumps virus or NDV, but will be agglutinated by the influenza viruses; etc. This relationship is not absolute but is dependent upon the amount of virus used and the length of time it is allowed to act upon the red cell.

Aside from its practical utility, the myxoviral hemagglutination reaction, and its mechanisms, are probably related to the initial stage in the proliferation of these viruses, e.g., adsorption to the surface of the host cell.

The Influenza Viruses

Influenza¹¹⁹ is a widespread disease of man occurring in sporadic interepidemic form, in periodic epidemics, and in pandemic form. Pandemics occurred in 1890 and in 1918–1919; in the latter the disease

occurred all over the world, and the estimated number of deaths was over 21 million. The epidemic periodicity differs somewhat between the two main types of influenza virus (see below), and epidemics of greater

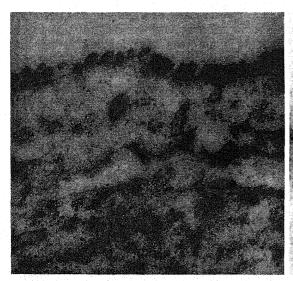
or lesser extent occur at two- to three-year intervals in the case of influenza A and at four- to five-year intervals with influenza B. The epidemic periodicity appears to be due to the accumulation of susceptibles, and the subtraction of immunes by death, in the population, but pandemic influenza seems to be a result of antigenic changes in the virus of sufficient magnitude that there is little effective cross-immunity between the pre-existing and new antigenic types.

The prevalence of the infection is indicated by the occurrence of demonstrable serum antibody in practically all adults and in most children over five years of age. It is probable that many infections are subclinical, or mild and regarded as common colds, and that the viruses persist in interepidemic periods in the form of such infections and as sporadic cases. There seems to be no unequivocal evidence of the existence of a chronic carrier state. Such persistence of these viruses perhaps contributes to the maintenance of observed antibody titers in the general population.

Morphology. 67, 133 The influenza viruses are morphologically indistinguishable, but there is some evidence that certain strains of influenza B are slightly larger than influenza A strains. As described elsewhere (Chap. Three), on primary isolation in the embryonated egg, and often for the first

few passages, the virus occurs as both spheres and filaments; both have viral activity, and there is some reason to believe that the spherical particles may differentiate from the filamentous forms. Spherical particles cut centrally in ultrathin sections are found to contain a central body 20 to 22 m μ in diameter which is surrounded by less dense material and a limiting membrane about 3 m μ thick.⁸⁸ Analysis of purified preparations has given a chemical composition of 60 to 70 per cent protein, 5 per cent carbohydrate, 20 to 30 per cent lipid, and about 1 per cent nucleic acid of the pentose type.

Toxin. 31, 74, 75, 87 Like certain other viruses, the influenza viruses are toxic in high concentration, and the toxicity persists after appropriate inactivation, as by ultraviolet irradiation, but is destroyed before hemagglutinin and antigenic activity. The toxicity is demonstrable by intracerebral inoculation in the mouse with symptoms referable to meningoencephalitic changes observed at autopsy. On intravenous or intraperitoneal inoculation the toxicity is pyrogenic in the rabbit, and fatal toxemia in the mouse is associated with liver and spleen damage with hyperemia, and with focal necrosis in the former and destruction of the malpighian bodies in the latter. The toxicity may be neutralized by antiserum



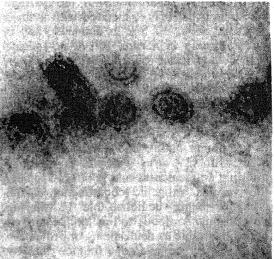


Figure 275. Ultrathin sections of entodermal cells of chorioallantoic membrane infected with influenza virus. Left, the virus particles in the form of spheres and short rods are on the surface of the cell, and the cell nucleus is below. \times 45,000. Right, a similar preparation at higher magnification showing the spherical bodies to consist of an internal body, a sharply defined dense membrane, and a less dense envelope. The obliquely sectioned rod appears to have an amorphous interior. \times 152,000. (Morgan, Rose, and Moore: J. Exp. Med.)

and is group-specific between influenza A and B with some strain differences observed. It is of some interest also that animals may be protected against the toxicity by pretreatment with neuraminidase, suggesting that the toxicity is related to attachment of the virus particle to the host cell.

Hemagglutinin.21 The red cells of many species of animals are so agglutinated, and those of the chicken, man, and the guinea pig are usually used. The hemagglutinin activity is intimately associated with the virus particle but may be separated from it by ether extraction. In fresh preparations the hemagglutinin activity parallels infectivity closely and is used as a measure of virus concentration. It is not, however, identical with infectivity; infectivity is destroyed at 42° C., leaving hemagglutinin activity unimpaired; after treatment at 56° C. the hemagglutinin is adsorbed on red cells but will not elute, and following treatment at 65° C. it is not adsorbed. The hemagglutinin of human strains is inactivated by trypsin, but that of swine influenza is not. The relative sensitivity of the human strains appears to be correlated with antigenic tvpe122 (see below).

Antigenic structure. Two kinds of antigens are present in these viruses. One is the soluble or S antigen, which is found free in infected tissue, is also present in the virus particle from which it may be extracted, together with hemagglutinin, by ether, and is irregularly separable from the particle by sonic lysis. It is associated with a small particle, about $10 \text{ m}\mu$ in diameter, and is nucleoprotein in nature. It is type-specific and demonstrable by complement fixation in the presence of antibody, and antibody to it is formed in response to infection, but not ordinarily in persons immunized with inactivated partially purified virus.

The second kind of antigen or V antigen is protein in nature and intimately associated with the virus particle. It contains multiple antigenicities and is strain-specific, the various antigenic components of the complex being demonstrable in varying proportions. Antibody to this kind of antigen is demonstrable as neutralizing or protective antibody, by complement fixation and by the hemagglutination-inhibition, or HI, reaction. The last serological reaction is simple to carry out. The hemagglutinating unit (HU) of virus is determined by serial dilution and is represented by the highest dilution pro-

ducing hemagglutination in 0.5 per cent erythrocyte suspension. Serial dilutions of the serum are titrated against four HU, and the end point is the highest dilution of serum that will prevent, or inhibit, the viral hemagglutination. The end point of hemagglutination, or hemagglutination-inhibition, may be measured with greater precision on the basis of optical density of the red cell suspension.⁸⁵

Nonspecific, i.e., nonantibody, inhibitors of a mucinous nature are often present in serum or tissue extracts, but only to low titer, and may be eliminated by pretreatment of the serum with RDE. Since the HI antibody is strain-specific and parallels, although it is not identical with, protective antibody, it is an extremely useful reaction for antibody surveys, is applicable to antigenic analysis of virus strains, etc.

It has long been known that host-specific antigen, derived from the host cell, is present in the virus. This kind of antigen is now established as occurring in the envelope in intimate association, but distinct from, hemagglutinin.⁶⁸

Antigenic varieties. The influenza viruses are separable into at least three main types which are immunologically distinct from one another, viz., type A, which was isolated in 1933; type B, isolated in 1940; and type C, isolated in 1949. Each of these types is made up of strains, related to one another by the type-specificity of the S antigen, but differing to a greater or lesser extent in the antigenic specificities contained in the V antigenic complex, and it has become almost axiomatic that a newly isolated strain will not be immunologically identical with strains isolated at other times. Such strain differences are demonstrable by the neutralization or protection test and by the HI reaction.

Type A influenza virus is the most common cause of influenza in man and shows the greatest strain variability. It has been possible, largely on the basis of the effectiveness of prophylactic immunity, to separate the multiplicity of type A strains into four main subgroups. The first of these is the swine influenza subgroup, strains of which were responsible for the 1918–1919 pandemic which persisted for perhaps 10 years as a cause of human influenza as indicated by serological evidence (see below). The first human strain to be isolated, the WS strain in 1933, is not identical with swine

889 VARIATION

influenza but is more closely related to it than subgroups found later. The second subgroup is typified by the PR8 strain of virus. The human strains isolated in 1933 to 1935 were of this subgroup and were responsible for type A influenza for another 10 years. The third subgroup is the A-prime (A')group, of which the FM1 strain is generally taken as a prototype. It appeared in 1946. and strains of this subgroup were responsible for human type A influenza through at least 1956. The fourth subgroup is that of the Asian strains which first appeared in 1957. This, in turn, has undergone antigenic variation.95 This time sequence in the appearance of antigenic subgroups is of particular significance in connection with naturally occurring antigenic variation (see below) and has practical consequences to effective prophylactic immunization.

Type B influenza viruses are also variable, but only two main subgroups are distinguished on the basis of the protective effect of immunization. The Lee strain is a prototype of the first of these, and Lee vaccine continued to give an effective prophylactic immunity until as late as 1954, although strains isolated somewhat earlier were found to be more distantly related to it. The second subgroup, representing successors to the Lee subgroup, has as its prototype the GL strains and additional antigenic variation has occurred. 109 Type C influenza viruses are less common than types A and B and appear to be more stable in that, while strain differences are apparent, these are not sufficient for the separation of subgroups, and in general the type C viruses are relatively homogeneous. The type C viruses have not been associated with epidemic disease in man, and, in fact, their relation to clinical illness is somewhat obscure.

Variation. There are three general kinds of variation among the influenza viruses, viz., variation in hemagglutinin species-specificity, variation in pathogenicity which perhaps includes hemagglutinin, and antigenic variation. There is also a variation between strains in their sensitivity to the nonspecific hemagglutinin inhibitor present in human and animal serums which appears to breed true even on continuous passage. On primary isolation the virus grows more readily in the amnionic than the allantoic cavity of the embryonated hen's egg and usually agglutinates fowl erythrocytes to a much lower titer than guinea pig or human O red cells, and the latter is expressed as the F:G hemagglutinin ratio. The primary isolate is said to be in the original, or O, state. After passage in the egg, the virus will grow in the allantoic cavity, is pathogenic for the mouse, and the F:G ratio approaches unity. Continued passage in the embryonated egg also often results in a rapid loss of virulence for man. When the virus shows these characteristics it is said to be in the derived, or D, state, and this change is the O-D variation which has its analogies with other microorganisms; the general phenomenon is discussed elsewhere (Chap. Seven).

Other changes may be induced by continuous passage, viz., the endotheliotropic variant produced by passage on the chorioallantoic membrane and the neurotropic variant resulting from passage in the mouse brain. As described elsewhere (Chap. Four), the inoculation of a nonadapted strain into the mouse brain results in an incomplete growth cycle with the appearance of noninfective or incomplete virus. There is also another kind of variation in hemagglutinin activity, characterized by partial or complete cross HI reactions in which three phases, designated P. O. and R. may be distinguished, which seems to be a consequence of differences in avidity of the hemagglutinin for antibody.

Antigenic variation. Variation in the constituent antigens of the V complex occurs with some facility under natural conditions, and similar changes may be induced in the laboratory. When the subgroups are separated, as noted above, the naturally occurring phenomenon appears to be a continuous variation in the antigenic character of type A virus, with occasional major changes of sufficient magnitude that there is no effective cross-immunity so far as man is concerned. The cross-reactions between major representative strains in the HI reaction are illustrated in the accompanying table. In the case of influenza B viruses, the second subgroup seems to have differentiated as a result of cumulative changes rather than a single major change. The evidence for this is of two kinds. One is the direct demonstration of antigenic differences in isolated strains, and the other is that of retrospective serological diagnosis in relation to age groups.

Surveys of serum antibody present in persons of all ages and sorted out with re-

Naturally Occurring Antigenic Variation Shown by Hemagglutination Inhibition	Naturally Occurring	Antigenic	Variation	Shown 1	by	Hemagglutination	Inhibition ⁸
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		RECIPROCAL OF TITERS†							
		***		туре А			ТҮР	E В	
	ANTISERUMS (FERRET)	Swine 1930	WS 1933	PR8 1934	FM1 (A') 1947	Asian 1957	Lee 1940	Todd 1950	
Type A	Swine 1930 WS 1933 PR8 1934 FM1 1947 Asian 1957	4096 32 <16 <16 <16	48 2048 32 <16 <16	<16 24 4096 <16 <16	<16 16 24 512 <16	<16 <16 <16 <16 256	<16 <16 <16 <16 <16	<16 <16 <16 <16 <16	
Type B	Lee 1940 Todd 1950	<16 <16	<16 <16	<16 <16	<16 <16	<16 <16	4096 64	<16 4096	

^{*}Compiled by Dr. Dorothy Hamre.

spect to specificity and age group reflect the prevalence in time of the subgroups of influenza A virus. In one such study completed in 1953, and later fully substantiated by studies both in this country and in England, it was evident that the serum of children contained antibody to the then only recently prevalent A-prime strains; that of individuals in the 15- to 28-year age group contained antibody to the PR8 subgroup as well; and antibody to the swine group, as well as to subsequently appearing antigenic varieties, was found only in persons at least 30 years of age. It is of special interest that, with the appearance of the Asian strains, antibody to this subgroup has been found in persons 70 to 90 years of age, suggesting that this apparently new antigenic variety may have been present in 1890.33, 108 The implication, of course, is that of the possibility of cyclic change, perhaps affected by human life span, rather than the appearance of new antigenic varieties in indefinite extension.

The validity of inferences as to a succession of antigenic varieties from the age distribution of serum antibody specificity is dependent upon the nature of the immune response in man.⁶⁴ It is suggested by data such as the foregoing, and has been substantiated experimentally, by immunization with monovalent vaccines of representative antigenic varieties, that (a) the initial or childhood experience reflects the dominant

antigens of the prevalent strains and is of limited effectiveness since the disease is most prevalent in this age group; (b) successive subsequent infections with differing antigenic varieties of the virus both reinforce the initial response and broaden it with the acquisition of new antibodies; and (c) the wide range of antibody activity in the serum of older persons is a consequence of contact with a broad range of influenza antigens with continuous reinforcement of prior antibody responses and the development of a progressively more effective immunity.

Antigenic variation may be produced under experimental conditions in a number of ways. Double infections with different virus strains result in recombination and the appearance of hybrids as well as parental types⁷⁶ (Chap. Seven) in the same way that bacterial virus hybrids are produced, and similar changes have been produced in mixtures of active and inactive virus which appear to be analogous to the bacterial and fibroma-myxoma virus transductions. Just as the antigenic structure of bacteria may be altered by growth in the presence of homologous antibody, influenza virus is similarly altered in the presence of antibody, both in egg culture and in infected partially immune animals, the changes in antigenic structure persisting in the absence of antibody. Representative changes produced by the latter method and demonstrable by the HI reaction are shown in the accompanying

[†]Homologous titers are shown in boldface.

INFECTIONS 891

Experimentally Induced Antigenic Variation Shown by Hemagglutination-Inhibition*

	RECIPROCAL OF TITERS†					
ANTISERUMS (FERRET)	PARENT STRAIN (PR8)	SECOND VARIANT (Ba)	FIFTH VARIANT (Fd)			
D						
Parent strain (PR8)	1536	48	8			
Second variant						
(Ba)	768	1024	128			
Fifth variant						
(Fd)	96	96	768			

^{*}Prepared by Dr. Dorothy Hamre.

table. The conventional interpretation is that such antigenic variants arise from mutants and are selected by the antibody present. It is possible that some such mechanism is operative in the appearance of the naturally occurring antigenic variants of influenza virus.

Infection in lower animals. Animals other than man are not, so far as is known, naturally infected with the human influenza viruses with the important exception of swine. Avian influenza viruses, including classic fowl plague, seem not to occur in man. 96

Swine influenza. This naturally occurring respiratory disease was observed in swine in this country at the time of the influenza pandemic in 1918. The etiology of the disease was described by Shope, who in 1930 isolated the swine influenza virus and a porcine variety of Hemophilus influenzae and showed that both kinds of microorganisms were required to produce the clinical disease. He later found a complex cycle of transmission, involving two reservoir hosts. The virus occurs in lungworms in infected swine, and the infected lungworm ova are excreted in the feces, ingested by earthworms, and the larvae develop in the earthworms. The infective cycle is completed when the infected earthworms are ingested by pigs, but the disease does not occur in the absence of some exciting cause, H. influenzae var. suis infection, but climatic conditions may apparently be effective also. The demonstration of the persistence of the infection in this masked form in intermediate hosts¹¹⁸ has been taken by some to suggest the possibility of the persistence of human influenza virus in some masked form, but there is no evidence to support such an inference.

There is little doubt that swine influenza virus is closely related to the influenza A virus strains prevalent during the 1918–1919 pandemic as indicated by serological studies such as those described above, but it seems likely that the animal infection was originally acquired from man.

Experimental infections. The ferret is highly susceptible to intranasal inoculation with the influenza viruses and may, in fact, be infected by contact. On primary isolation of the virus from man, there may be few symptoms of disease in the ferret, but the infection is demonstrable by a rise in antibody titer. On serial passage, virulence increases and there are overt symptoms of disease and lung consolidation. Similarly, the mouse lightly anesthetized with ether may be infected in primary isolation of the old type A strains by intranasal inoculation: fresh isolates usually do not produce lung lesions, but pulmonary consolidation is produced by passage strains of the virus. The Aprime and Asian strains are much less virulent for the mouse, and this animal is not practical for primary isolation of them. Type B influenza virus generally grows poorly in the mouse, even on prolonged passage. The embryonated hen's egg may be infected by various routes, but isolation is most often successful on inoculation of the amnionic cavity of 12- to 14-day old eggs. On passage the virus will grow in the allantoic cavity, in the yolk sac, and on the chorioallantoic membrane. Of these methods of isolation, inoculation of the amnionic cavity of the embryonated egg is regarded as one of the most sensitive.

The disease in man.³² The virus enters the body via the respiratory tract, and the incubation period of influenza is short, one or two days, giving the epidemic and pandemic disease its explosive character. Symptoms referable to the respiratory tract, including nasal, pharyngeal, and laryngeal irritation and associated symptoms, occur but are often subordinate to the constitutional reaction with fever and prostration. The syndrome is variable and not characteristic, and influenza cannot be diagnosed on clinical grounds.

[†]Homologous titers are shown in boldface.

The most common complication, and usually the immediate cause of death in the fatal disease, is pneumonia resulting from secondary bacterial, most often staphylococcal, but also streptococcal or pneumococcal, infection.^{65, 97, 116} Uncomplicated influenza may, though rarely, assume a fatal fulminating form, ⁹¹ and in such instances there is intense congestion of the trachea with consolidation and hemorrhage in the lungs, but little evidence of cellular reaction.

Following an epidemic, the disease becomes apparently less prevalent, but this is a consequence of the occurrence of an increasing proportion of clinically mild infections. Serological studies have shown that the incidence of infections continues, with persistence during interepidemic periods in smoldering endemic form, 62 recurring in severe form when antigenic variation has resulted in the appearance of strains differing sufficiently that the existing immunity is less effective.

Immunity. Recovery from the disease results in the appearance of antibody to both S and V antigens giving complement-fixing, neutralizing, and HI reactions. Effective immunity is associated with antibody to the strain-specific V antigen. It persists for perhaps a few months, and there is evidence suggesting that it may be dependent upon the presence of neutralizing antibody in the respiratory mucosa.

The immunity resulting from initial exposure to the viral antigens appears to be of a low order but, as described above, seems to increase in effectiveness with repeated exposure to the virus so that the total experience of perhaps three decades provides a reasonable measure of effective immunity. This often, but not invariably, modifies subsequent infections so that the disease is subclinical or little more than a mild coryza. Such immunity is not effective against virus strains in which major antigenic changes have occurred.

Laboratory diagnosis.^{23, 78} The differentiation of influenza from a variety of clinically similar conditions is etiological and requires direct or indirect identification of the virus in the laboratory. Direct identification is accomplished most readily by the inoculation of nasal washings, containing appropriate antibacterial substances such as a mixture of penicillin and streptomycin, into the amnionic cavity of the 12- to 14-day

old embryonated egg. Virus is present in the amnionic fluid in two to four days, may be detected by the agglutination of type O human erythrocytes, and may be identified by the HI reaction with known antiserum. The virus may also, but with less certainty, be isolated by intranasal inoculation of the ferret and subsequent demonstration of a rising antibody titer or the presence of the viruses in the lung tissue.

The presence of the virus is shown indirectly by a rising antibody titer in paired serum specimens, the one taken as early as possible in the acute stage of the disease, and the other 10 days to three weeks later. The complement-fixation and HI reactions may be used; the former is less likely to be affected by minor antigenic differences between the test antigen and the infecting virus. A four-fold or greater rise in titer is regarded as diagnostic.

Prophylactic immunization. Prophylactic immunization against the disease is carried out with inactivated virus grown in the allantoic cavity of the embryonated egg and partially purified, commonly by adsorption and elution from chicken red cells. An appreciable degree of effective immunity against homologous or closely related strains of virus is apparent in about a week and persists for some months. The degree of protection conferred is dependent upon the time elapsing between immunization and exposure, and it is estimated that 40 to 70 per cent of persons are protected. 117, 120

Because of the changing antigenic character of virus strains, the vaccine strains are subject to alteration. Vaccine is usually multivalent, containing both type A and type B virus strains, but the type A has been changed from the PR8 strain to A-prime strains such as FM1, and later to include Asian strains, while the type B strains have shifted from the group having the Lee strain as a prototype to later-appearing types such as the GL or Todd strains. As prepared commercially, the vaccines are kept in monovalent form and mixed to give polyvalent vaccine of a composition determined by the nature of current isolates.

The use of living vaccines, consisting of virus adapted to mammalian cells in culture and administered intranasally, has been of interest in Russia and has also been tested in England, but as yet has not proved to be markedly superior to the inactivated virus.

The Influenza-like and Other Respiratory Viruses

A number of viruses other than the influenza viruses have been isolated from a variety of acute respiratory diseases lacking precise clinical characterization and considered to be common colds, pneumonitis, etc., and on occasion, from apparently normal individuals. Some appear to be related to the influenza group, others make up the distinctive group of adenoviruses (see below), and still others are miscellaneous in the sense that they are unrelated to one another or to established groups of viruses. Antibody surveys have indicated, however, that they may be more prevalent than the frequency of isolation would suggest, and their occurrence emphasizes the diverse etiology of acute respiratory disease. 121

PARAINFLUENZA VIRUSES

The viruses now considered to make up the parainfluenza group account for a considerable portion of ARD. Isolated from upper respiratory tract disease of varying severity, they account for much of such disease in children^{26, 27} and are by no means unknown in adults. 19 They are found in this country, in Europe, 123 and in Australia and are no doubt world-wide in occurrence. Although of varied etiology, the disease is substantially the same, resembling a common cold with pharyngitis, rhinitis, bronchitis, and fever, lasting three to four days,94 and tending to be more severe in children. The etiological relation of these viruses to such upper respiratory disease is indicated not only by specific antibody response, but also by experimental reproduction of the disease in human volunteers. 130, 131

It has been observed that when guinea pig erythrocytes are added to monkey kidney cell cultures of influenza virus, the red cells are aggregated about and adsorbed by the cells in which viral proliferation is occurring, as noted above. This is not a unique property of myxoviruses for it occurs also with certain simian viruses and PPLO in cell culture. Certain of the parainfluenza viruses isolated by application of this technique have been called hemadsorption, or HA, viruses. These and similar viruses, described under various names, and se-

rologically distinct, or even unrelated, to one another and to the influenza viruses have been arranged as types of parainfluenza virus.⁸

Parainfluenza 1. This designation includes two viruses which are immunologically related but not identical, the Sendai virus and the HA 2 virus.

virus. (Newborn pneumonitis Sendai virus, hemagglutinating virus of Japan or HVJ, influenza virus type D). This virus, has been isolated repeatedly from both human and animal (swine, mice) sources in Japan since 1952. It appeared to be the etiological agent of highly fatal epidemic pneumonitis of newborn children in Japan and has been found in infants in Moscow and in Germany. It was also associated with an epidemic of influenza in Vladivostok in 1956. It was isolated from affected individuals during the epidemic, and serum antibody to it persisted for some months in the local population.

The virus was originally isolated in the mouse lung, where it produced influenzalike lesions, e.g., consolidation, and is cultivable in the embryonated egg. It grows well in the allantoic cavity, and also on the chorioallantoic membrane where it produces small pock-like lesions or plaques similar to those produced by herpes simplex virus. It produces a hemagglutinin, agglutinating human, chicken, guinea pig, monkey, and mouse erythrocytes, and is serologically related to the influenza viruses, more closely to type B than the other types, and has been called influenza virus type D.

Significant antibody titers to the Sendai virus have been observed in this country. In convalescents elsewhere the HI titers reach levels of 1:32 to 1:64, the complement-fixing antibody titers 1:32, and the neutralizing antibody titers 1:128 to 1:512. In comparison, HI titers of 1:32 or higher were found in 52 per cent of 118 individuals under 18 years of age, and in 37 per cent of 248 individuals over 18 in this country, suggesting a relatively high prevalence of immunizing infection. The virus is, however, related to mumps virus, and some of the observed antibody titers may be a result of prior mumps infection. Four-fold or greater rises in HI antibody titer have also been observed in this country in association with

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influenza-like disease and with a few cases of infectious mononucleosis.

HA 2 virus. Hemadsorption type 2 virus, together with HA 1, was isolated by the technique described above from children with febrile respiratory disease.26 Essentially no cytopathic effect was apparent in monkey kidney cell culture, although hemadsorption occurred, but in later passages there was a partial cytopathic effect with the appearance of scattered round cells and eventually focal degeneration in the sheet. The culture fluid contains hemagglutinin, and complement-fixing antigen may be prepared from sonic lysate of the culture. Some of the strains will grow in the amnionic cavity of the seven-day embryonated hen's egg. This virus has also been isolated from adults³⁷ as well as repeatedly from children here and in Japan. Substantially the same virus has been isolated in Denmark, where it was called Copenhagen 222. Infections with this virus account for a small portion of adult ARD, in one study the figure given is 2.6 per cent, but it has been reported that 84 per cent of American adults have antibody. The last is taken to suggest that the virus is widely disseminated. The independence of the Sendai and HA 2 strains is indicated by a lack of correlation in antibody found in surveys. The virus is not antigenically related to influenza, mumps, RS or ECHO 28 (Chap. Thirty-nine) viruses. The pathogenicity of the cultured virus has been demonstrated by the inoculation of human volunteers to produce respiratory

Parainfluenza 2.24 (Croup associated or CA virus, acute laryngotracheobronchitis or ALTB virus). This virus was isolated from two of 12 children suffering from acute laryngotracheobronchitis, or croup, by culture in monkey kidney tissue culture. A cytopathogenic effect appeared in three to five days and consisted of the formation of a syncytial mass with loss of cell boundaries and the appearance of vacuoles and granules in the cytoplasm. It has subsequently been isolated both in this country and in England.

This virus forms a hemagglutinin for chicken and human erythrocytes which has the unusual characteristic of producing agglutination at 4° C., but not at 37° C.; in fact, the process was repeatedly reversible by alternate warming and chilling. Red cells treated with RDE were inagglutinable, but after treatment with CA virus were still

agglutinated by influenza viruses types A and B and by Newcastle disease virus. The virus particle was found to be 90 to 135 m μ in diameter.

In five of 11 children with the disease, a rise in antibody titer to this virus was observed. A small number of normal persons were examined, and significant titers of neutralizing antibody were found in four of 16 individuals 34 months old or less and in 19 of 20 individuals 21 to 30 years old. In contrast to the more or less constant prevalence of the other parainfluenza viruses, type 2 seems to be variable in its occurrence; 22 per cent of American adults are reported to have antibody to it.

Parainfluenza 3. This virus was originally described as hemadsorption virus type 1, and was isolated, together with HA 2, from children having acute febrile respiratory disease. It has subsequently been recovered from both children and adults and is apparently widely disseminated, since antibody has been found in 100 per cent of adult Americans examined.

A hemadsorption virus was isolated from cattle suffering from shipping fever (which is also possibly of Pasteurella etiology [see Chap. Twenty-five]) and designated SF-4, and there is serological evidence of the prevalence of the infection in sheep.⁴⁶ This virus is serologically closely related to parainfluenza 3 virus strains of human origin. The relationship is so close that the two appear identical by the conventional serological methods, but small differences are apparent in the immune response of the guinea pig to intranasal inoculation.² Apparently the same bovine virus has been found in Sweden¹³ and serological studies have indicated that the bovine infection is widespread, with 70 per cent or more of cattle giving positive reactions.3,14 It is not known what relation there may be between the bovine and human diseases. An additional serotype, designated type 4, has been isolated which apparently occurs as at least two subtypes.22

Other myxoviruses. Other viruses, overtly similar, but of as yet uncertain status, have been described. An agent associated with human croup and designated U virus has been reported⁹⁸ which resembles parainfluenza 2 in that hemagglutination occurs more readily in the cold than at incubator temperatures. Another virus, the SA virus, has been isolated from hamster brain fol-

lowing inoculation with chick embryo allantoic culture of nasal washings from an individual with acute upper respiratory infection. 115 The hemagglutinating activity for guinea pig erythrocytes is similar to that of myxoviruses, and the cytopathic effect on monkey kidney cultures is that of syncytium formation. It is apparently unrelated antigenically to other known viruses. Still another antigenically unrelated virus with the properties of a myxovirus was isolated71 from a number of persons with upper respiratory illness in monkey kidney cell culture. The cytopathic effect is one of round cell formation, and the virus, designated M-25, is antigenically unrelated to the other myxoviruses. The virus described by French workers as EA 102 isolated from acute febrile respiratory illness in children²⁸ is characterized by a syncytial cytopathic effect in KB cell culture, and is serologically related to the HA viruses, but does not form a hemagglutinin. The status and significance of virus isolates such as the foregoing is uncertain.

RESPIRATORY SYNCYTIAL VIRUS 126 (RS Agent)

This virus, also known as the chimpanzee coryza agent or CCA, was first isolated from chimpanzees having symptoms of respiratory disease, and spontaneous infection occurred in a laboratory worker.

The virus was originally grown from throat swabs on human liver tissue culture, with cytopathology apparent in about a week. This virus did not infect the common laboratory animals.

The virus has subsequently been isolated from children with bronchopneumonia and bronchitis rather than croup,^{7, 18} and in adults as a natural infection associated with mild upper respiratory disease.⁷² Antibody surveys have indicated that the human infection is acquired early in life; in one survey the proportion of individuals with antibody was found to be 8 per cent in children six to 12 months old, rising to 84 per cent in the 15- to 29-year age group, and falling off to 68 per cent in those 50 years or more old.⁵⁸

RS virus is an ether-sensitive RNA virus. 90 to 120 mu in diameter, but differs from the myxoviruses in that it is not cultivable in the embryonated hen's egg and is not hemagglutinating. It is unrelated antigenically to myxoviruses, and antigenic subtypes have been differentiated.29, 137 It is, however, closely similar morphologically to the myxoviruses10, 20 and, like the paramyxovirus subgroup, produces syncytial CPE in cell cultures. It is, therefore, considered to fall into the paramyxovirus group with the parainfluenza viruses. 134 A closely similar virus, but producing a different and unusual CPE, has been isolated from young adults suffering from mild upper respiratory disease.59

The Mumps and Related Viruses

The mumps virus, the Newcastle disease virus (NDV), and the virus of fowl plague are related to the influenza viruses with respect to hemagglutinin activity and affinity for the receptor substance of the erythrocyte and are members of the paramyxovirus group. In the receptor gradient order of inactivation of receptor sites on the red blood cell, the mumps virus and NDV occur at one end of the series; viz., red blood cells treated with mumps virus will still agglutinate with NDV and influenza A virus, those treated with NDV will agglutinate with influenza A virus but not with mumps virus, and those treated with influence

enza A virus will no longer agglutinate with either mumps virus or NDV.

MUMPS

Mumps, or epidemic parotitis, is a highly contagious, widespread, but seldom fatal disease of man that has long been known because of its distinctive clinical character, *i.e.*, swelling and tenderness of the parotid glands and orchitis in a variable but appreciable portion of cases. Some 60 per cent of adults are immune as compared with

90 per cent immune to measles virus. The disease tends to occur in the winter and early spring, with an epidemic periodicity of seven to eight years.

The mumps virus is present in the saliva and may be isolated within the first few days of the disease from the urine. 132 It may be grown in a number of kinds of cell culture. the cytopathic effect differing with the virus strain and kind of cell culture; e.g., on isolation in HeLa cell or monkey kidney cell culture foci of granular degeneration occur, but with adapted strains other effects are produced, varying with the type of cell culture.⁵⁶ Estimations of particle size have varied considerably; a size range commonly given is 90 to 135 mµ, but values as high as 340 m μ have been recorded. In electron micrographs the observed size of the particle varies, and it is probable that infectivity may be associated with particles of different size. It resembles the influenza viruses not only in hemagglutinating activity as noted above, but also in its hemolytic activity in high viral concentration and the occurrence of two kinds of antigens. The S, or soluble, antigen is separable from the infectivity by differential centrifugation and fixes complement in the presence of antibody, and the V, or viral, antigen is found in the infective particle. Infectivity is destroyed by standing at room temperature for a few days or by 0.1 per cent formalin or ultraviolet irradiation with persistence of hemagglutinin and S and V antigenic activity, and the S antigen is relatively thermostable. The V antigen is related to that of the Sendai (parainfluenza 1) virus.⁵⁰

The disease in man. This disease occurs only in man, and there is no animal reservoir of infection, although several species of monkeys may be infected experimentally. The incubation period is about three weeks. A febrile reaction may or may not occur and is followed within 24 hours by enlargement of the salivary glands, usually the parotids but the sublingual and submaxillary glands may be affected also, and there is an edematous infiltration of the adjacent tissues. The swelling may be bilateral, unilateral, or unilateral followed by involvement of the unaffected side within a few days. It reaches a maximum in 48 hours and persists for one to two weeks, though some evidence of swelling may persist for longer periods. Orchitis occurs in perhaps 20 per cent of cases, but the proportion may vary considerably from one outbreak to another, and the involvement is bilateral, in only 15 to 20 per cent.

Symptoms referable to the central nervous system, indicative of meningoencephalitis, occur in from less than 1 to as much as 10 per cent of cases; such cases occurring in the absence of parotitis are often classed as aseptic meningitis. The pancreas, epididymis, prostate, and ovaries are affected only relatively rarely, although some degree of pancreatitis may be more common than generally thought. Involvement of tissues other than the salivary glands may occur prior to or after the parotitis, or even in the absence of parotitis. Epidemiological evidence, supported by a certain amount of experimental data, indicates that a considerable portion of infections may be asymptomatic but, as indicated above, the infection is less prevalent than measles.84

Immunity. Strains of mumps virus appear to be of the same antigenic type, and recovery from the disease results in a solid and lasting immunity, possibly reinforced from time to time by reinfection, and unquestionable second attacks are quite rare. The immunity is demonstrable as complement-fixing, HI, and protective serum antibody and a hypersensitivity giving the delayed type of reaction to intradermal inoculation of inactivated virus.

The complement fixation test is subject to refinement⁴¹ if both S and V antigens are available, because antibody to the S antigen appears earlier, within a few days of onset, and may reach high titer before antibody to V antigen is detectable. Antibody to the S antigen disappears more rapidly, leaving persisting antibody to the V antigen as evidence of past infection.

A fourfold or greater rise in complementfixing antibody titer between paired serums, taken as early as possible in the disease and two to three weeks after onset, is regarded as diagnostic. Serological diagnosis is not important when the syndrome is typical but is of value in meningoencephalitis without involvement of the salivary glands. The hypersensitivity appears too late in the disease to have diagnostic value, and this test should, in fact, be avoided because the intradermal inoculation of inactivated virus often results in increased titers of antibody to the V antigen. Some convalescent serums give cross-reactions with NDV, and with Sendai virus. A skin test for hypersensitivity to the virus has been of interest, but its correlation with an effective immunity is as yet uncertain.⁴⁹

There has been some interest in prevention or modification of the disease, especially with reference to orchitis in individuals past puberty, by the use of pooled γ -globulin or convalescent serum. Such passive immunization has, however, given only questionable results. Active immunization of monkeys and of man with egg culture virus inactivated with ether or ultraviolet radiation has been partially successful. In man a three-fold difference in the incidence of the disease has been observed between immunized and control groups. 63

Experimental infections. Mumps may be reproduced in several species of monkeys by direct inoculation of the gland via Stensen's duct and the infection passed in these animals by inoculation with suspensions of parotid gland tissue. The experimental disease is similar to that in man, with occurrence of parotitis, facial edema, and associated pathology. Suckling mice and suckling hamsters may also be infected, and sufficient infection occurs in the lungs of adult hamsters to induce antibody formation.

NEWCASTLE DISEASE VIRUS⁶¹

Newcastle disease (pseudo fowl pest, pseudo poultry plague, avian pest, avian distemper, avian pneumoencephalitis) is a disease of birds which is occasionally acquired by man through contact with infected birds. The natural hosts include domestic fowl such as chickens, turkeys, guinea fowl, and parrots and wild birds such as sparrows and pigeons. There is some reason to believe that it may have originated in Indonesia, where it was first described in 1926. It appeared in England in the same year and was eradicated by slaughter and terminal disinfection. It has since been reported from all over the world. It was first identified in this country in 1944 but may have been present as early as 1935. The virus tends to be pleomorphic and varies widely in its pathogenicity and tissue tropism, giving an impression that it may be evolving rapidly. It has been of particular interest as a viral agent that may become more closely adapted to man.

Morphology and cell culture. The virus

particle is 80 to 120 m μ in diameter, but is not necessarily spherical, and may be filamentous or sperm-like in shape. The latter form has been reported as having a head 70 by 180 m μ and a tail as long as 550 m μ . It is unusually resistant under both natural and laboratory conditions. In the first instance, poultry quarters may remain infective for many weeks and produce disease in freshly introduced chickens. Under controlled conditions, some strains of the virus have been found to remain infective after exposure to 56° C. for as long as 180 minutes. In dried secretions and tissues viral activity persists for many months, and the virus has substantial resistance to disinfectants such as formalin and phenols, and glycol aerosols have failed entirely to prevent transmission of airborne infection.

The virus is readily cultivable in the embryonated egg and in several kinds of tissue cultures. When it is grown in the allantoic cavity, hemagglutinin is present in the allantoic fluid which falls between those of mumps virus and influenza A virus in the receptor gradient series noted above, and on this basis NDV is regarded as a member of the myxovirus group. Heavily infected allantoic fluid is also toxic, killing mice in one to three days after intravenous inoculation. Intranasal inoculation in this animal produces interstitial pneumonia that is a manifestation of toxicity, for the virus does not multiply in the mouse lung.

Disease in chickens. After an incubation period of four to 12 days, the onset of the disease in chickens is sudden and characterized by drowsiness, rapid respiration, and fever. Even before symptoms appear, the virus is widely distributed in the body. in highest concentration in the lungs and spleen. There is mucous discharge from the beak and nose, diarrhea, cyanosis, and respiratory distress, followed by convulsions and paralysis, with death in about a week. On autopsy multiple necrotic foci are found in the viscera, there are interstitial pneumonia, hemorrhagic patches in the gastrointestinal tract, and localized meningoencephalitis with degeneration and necrosis and hemorrhage. This syndrome is subject to some variation, the respiratory and gastrointestinal symptoms being associated to some degree with Old World virus strains, while the pneumonic and encephalitic manifestations are often predominant in infection with American strains.

The disease is by no means invariably fatal, and, in fact, virulence varies widely between virus strains. Of two strains isolated in this country, for example, one killed 90 per cent or more of naturally infected chickens, while the other killed only 1 per cent. Under experimental conditions, the LD_{50} dose of cultured virus has been found to vary among strains from 0 to 10^8 per 0.5 ml. of allantoic fluid.

Diseased birds are highly infectious, shedding large amounts of virus in secretions and excretions. This, coupled with the resistance of the virus to drying, tends to favor the transmission of variants of the virus. The infection appears to be transmitted largely by aerosols of dried infectious material, but direct contact with diseased birds or infected carcasses may also be a source of infection. Although the virus is present in the blood, there is no evidence that biting arthropods transmit the infection.

Immunity. While there seem to be minor antigenic differences between strains of the virus, chickens recovered from the disease are solidly immune to reinfection. Virus strains of low virulence are, in fact, used as immunizing agents, and are often administered as aerosols. The immunity is associated with the presence of serum antibody. It is titratable as complement-fixing antibody, neutralizing antibody, antitoxic antibody, and HI antibody. Very high titers are found two to three weeks after initiation of the infection, and HI titers of 1:1280 and neutralizing antibody titers of 1:1 million are not uncommon.

Experimental infections. Guinea pigs, rabbits, and pigs are resistant to infection, the pneumonia produced in the mouse is not transmissible in series, but the ferret responds to intranasal inoculation with the production of antibody. Mice, hamsters,

cotton rats, and rhesus monkeys may be infected by intracerebral inoculation to produce meningoencephalitis of increasing severity with passage.

The disease in man. Human infection is not common and appears to be confined almost exclusively to those who are closely associated with poultry, and laboratory infections have occurred. The incubation period is very short, two days or less, the disease is a unilateral conjunctivitis without corneal involvement, and there may be preauricular adenitis, malaise, and chills, but without fever. No fatal cases have been reported, and recovery is complete within two weeks.

Serum antibody may or may not be detectable after recovery, and serodiagnosis is complicated by the presence of nonspecific neutralizing activity in the serum. One of these is heat-labile and destroyed at 56° C. in 30 minutes, and the other is stable to such heating and associated with neutralizing antibody to mumps virus as noted above. It is supposed that at least some strains of mumps virus have antigens in common with NDV. In general, serodiagnosis of the human infection is not dependable.

The number of persons exposed to infection is very large. It is estimated that seven to eight million people are intimately associated with the raising and care of chickens in this country, and several hundred thousand others are concerned with the processing of poultry. All human cases found thus far have been initial infections acquired from infected birds, and transmission from man to man has not been observed. Neither has there been an instance of human infection acquired from dressed poultry. It is highly problematical whether strains of the virus of higher virulence for man may appear.⁶⁰

The Measles (Rubeola) Virus

Measles is one of the commonest diseases of childhood, and the median number of cases reported each year in this country exceeds half a million even though many cases are probably not reported. Man appears to be universally and highly susceptible to this infection, and the disease confers a solid immunity. These two factors combine to make measles the classic ex-

ample of periodicity in the prevalence of infectious disease (Chap. Ten), and it tends to recur in epidemic form at 2- to 3-year intervals. The probability of acquiring the infection is sufficiently great that the disease occurs early in life in most instances, but some individuals escape childhood infection, as in rural or other areas where contacts are less numerous, and the disease

may appear in young adults aggregated in large groups, as in military camps.

Morphology and cell culture. 12 The viral etiology of measles has been known since 1911, when the filterable nature of the infectious agent was demonstrated, and studies with gradacol membranes indicated a particle diameter of about 140 mu. It was grown in chick embryos and chick embryo cell cultures, but with no CPE. It remained poorly known because of the necessity for infectivity assay in monkeys, which are now known to be often immune, until it was isolated by Enders and his co-workers in 1954 in human and monkey kidney cell cultures in which a characteristic CPE is produced. The virus grows in human amnion cells on primary isolation, and has been adapted to a variety of cell lines.81

The virus is, like the other myxoviruses, an ether-sensitive RNA virus, the type of nucleic acid being inferred from the failure of halogenated deoxyuridines to inhibit growth in cell culture, since the uridine compounds are considered to be specific inhibitors of DNA synthesis. The virus particle has helical symmetry, the helices of the central core having a diameter of 16 m μ and a subunit periodicity of 4.5 m μ , with radial projections into the outer envelope in the mature particle. The measles virus thus closely resembles the

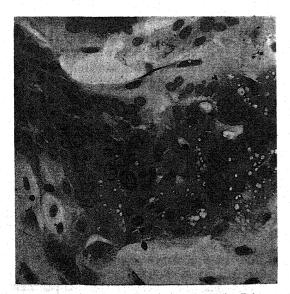


Figure 276. Measles virus in human renal cell tissue culture after 20 days' incubation showing multinucleated giant cell formation and nuclear acidophilic masses. (Enders and Peebles: Proc. Soc. Exp. Biol. Med.)

parainfluenza and other paramyxoviruses in morphology.

In cell culture the CPE is syncytial in character, with the development of eosinophilic cytoplasmic inclusion bodies, the latter providing the basis for the earlier tentative grouping of this virus with the herpesviruses. Complement-fixing antigen is found in culture fluids. The virus is hemagglutinating for monkey cells, and the hemadsorption phenomenon occurs in cell culture sheets. Hemagglutinin-inhibiting antibody occurs in antiserums, and the cell culture system is used for the titration of neutralizing antibody. The virus appears to be antigenically homogeneous, and its marked stability in this respect is indicated by the persisting effective immunity as well as by the failure to find antigenic variants.

The measles virus is closely related to the viruses of canine distemper and rinderpest antigenically, and indistinguishable from them in morphology and CPE, but the latter two viruses are set apart from the measles virus by failure to show hemagglutination. Distemper is a disease of carnivores, including foxes, wolves, and ferrets, as well as the dog, while rinderpest is a disease of cattle and swine. The antigenic interrelations are such that man infected with measles virus produces both distemper and rinderpest neutralizing antibody, infected with rinderpest produce measles neutralizing antibody, but immunization of cattle with measles virus does not protect against rinderpest challenge. Animals infected with distemper virus produce little or no measles neutralizing antibody, but dogs hyperimmunized with measles virus produce antibody to distemper virus. 107

The disease in man.¹²⁸ The infection is probably acquired through the oropharyngeal and ocular pathways.¹⁵ Viral replication occurs in localized areas in the lymphatic system, followed by a transient viremia, during which the virus may be isolated from the blood, and by the appearance of symptoms.

The disease is initiated by prodromal symptoms of a few days' duration which are essentially catarrhal in nature, and include cough and fever. During this period, characteristic lesions, Koplik spots, appear on the buccal mucosa. There are focal exudations of serum and epithelial cells that form vesicles which become necrotic, and their gross appearance is that of a white or bluish white center on an erythematous area.

Koplik spots are diagnostic of measles and occur in 95 per cent or more of cases. Virus is present in the nasopharyngeal and tracheobronchial secretions during this period and for a day or two after the appearance of the rash.

The characteristic macular or maculopapular rash, tending to be confluent, appears within one or more days and spreads over the entire body. The capillaries of the corium are affected first, there is proliferation of epithelial cells, a serous exudate spreads into the epidermis, there is necrosis of epithelial cells, and the rash becomes vesicular. It becomes brownish in a week or 10 days, followed by desquamation.

During the course of the disease there is a general lymphoid hyperplasia, and multinucleate giant cells are found in the tonsils, adenoids, lymph nodes, spleen, and appendix, and in the alveolar and bronchial epithelium in interstitial pneumonitis and what has been referred to as giant cell pneumonia.69 Two kinds of giant cells are found, the Warthin-Finkleday type in the lymphatic tissue, which has nuclei resembling those of lymphocytes and does not contain inclusion bodies, and the syncytial-epithelial type, which may contain as many as 40 nuclei with both cytoplasmic and nuclear inclusion bodies. There is a reduced resistance to secondary bacterial infection,

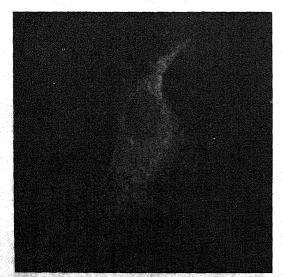


Figure 277. Intracellular measles virus in human epithelial cell tissue culture, treated with convalescent serum and with fluorescein-labeled antiglobulin serum. × 480. (Rapp and Gordon.)

especially with hemolytic streptococci. Encephalomyelitis, with demyelination in the brain and cord, is an uncommon complication, with a case of fatality of perhaps 10 per cent, but more than half the survivors are left with some impairment. Chemotherapy is useful only in minimizing secondary bacterial infection.

Immunity. Antibody is not detectable prior to the appearance of the rash, but thereafter the immune response is very rapid, with the appearance of significant titers within 48 hours. Recovery from the disease is associated with a solid immunity, no doubt periodically reinforced by subsequent contact with the virus, which persists essentially throughout life. Immunization by infection occurs early in life, with perhaps 20 per cent immunes among preschool children, and the proportion rises rapidly to 95 per cent at eight to nine years of age. Complement-fixing, HI, and neutralizing antibody, though maximal in convalescent serums, is present in almost all adult serums.

Immunization. Passive immunization with pooled γ -globulin following exposure to infection may abort or modify the disease, depending upon how soon it is administered; if the disease is aborted there is no active immune response. Pooled γ -globulin, *i.e.*, from adult serums, contains neutralizing antibody to titers of 1:1000 to 1:2000, and HI antibody to titers of 1:128 to 1:51286 which may persist for as long as seven years after the expiration dates of the preparations.

Active immunization.⁴² Attempts to immunize against measles were initiated as early as 1758,³⁸ but it was not until the development of methods of virus culture noted above that the preparation of vaccines became practical. Two kinds of vaccines have been developed, the one inactivated unmodified virus, and the other attenuated living virus.¹²⁷ A number of attenuated strains have been developed by adaptation to growth in various kinds of cell cultures, of which the Edmonston B strain of Enders has been widely used in this country.

Immunization with inactivated virus gives little untoward reaction, and the vaccine is given in multiple doses, usually three, at monthly intervals. In one study⁴⁷ it was found that the vaccine was 82 per cent effective in preventing any evidence of measles and 93 per cent effective in preventing unmodified measles during an epidemic

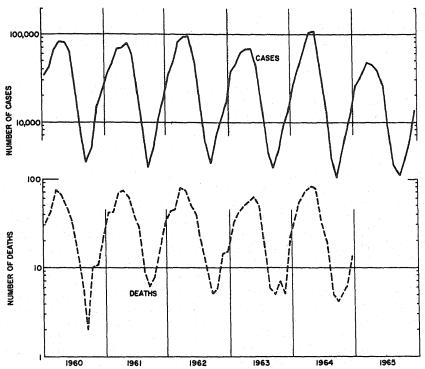


Figure 278. Cases of and deaths from measles reported in the United States during the period 1960–1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare.)

which occurred seven weeks after completion of immunization. Follow-up for longer periods, including serum antibody titrations, has shown, however, that the immunity so produced tends to be temporary and disappears in a year or two.¹⁰⁰

Immunization with living attenuated virus produces reactions in the form of the mild disease; further attenuation may result in loss of immunogenic potency. Such vaccines have been given alone, with tolerated reactions, or in combination with γ -globulin, to give conversion rates, *i.e.*, appearance of serum antibody, of 97 to 99 per cent. 90 Attenuated virus vaccine has also been given following inoculation with inactivated

vaccine to give an equally satisfactory immune response. Active immunization against measles is now beginning to be widely applied, and its established efficacy more precisely evaluated.

be transmitted to monkeys, both Indian (Macacus rhesus) and South American (M. cynomolgus), by inoculation by various routes, with blood or pharyngeal washings taken early in the disease, but the susceptibility of such animals is irregular. This variability is now known to be a result of the occurrence of immunizing disease in these animals, many of which are found to be immune.

The Rubella Virus 82, 92, 111, 129

Rubella, or German measles, is an exanthematous disease of childhood which resembles measles in many respects but is milder, e.g., the catarrhal symptoms of the prodromal stage are less severe, the subsequent febrile reaction is slight, and the rash

is macular, discrete, or confluent, resembling that of early measles. Koplik spots are not present, and there is characteristic lymphadenitis affecting the cervical and occipital nodes with swelling persisting for two or three weeks. Because of its mild nature it is

not an important pediatric disease, but it has assumed much greater significance in female adults in the child-bearing age group because of its teratogenic effects on the fetus. The association of infection of the mother during the early months of pregnancy with a wide variety of malformations, as well as with abortion and stillbirth, was noted by Gregg in Australia in 1941 and has since been amply confirmed by many observers.

The disease has been assumed to be of viral etiology, but convincing evidence was lacking until about 1962, when several methods of detecting and demonstrating the virus were developed. The fortuitous epidemic prevalence of the disease in the United States and England in 1964-1965 made possible considerable advances in knowledge.

Cell culture. Propagation of the virus in cell culture often produces no CPE. Demonstration of the presence of virus in such cultures is dependent upon its interference with secondary infection with a variety of other viruses, usually of the enterovirus group, which produce CPE; i.e., failure to infect the cell culture with the CPE-producing virus indicates the presence of rubella virus. ECHO-11 was originally used, Coxsackie A-9 has been used frequently, and a number of other viruses may be used also. 93 This indirect, or interference, method may be quantitated to allow the titration of rubella virus as tissue culture interference doses (TCID), and the system is adaptable to the titration of neutralizing antibody. A variation of the interference assay has been described in which Newcastle disease virus, a hemadsorption virus, is used, followed by treatment with bovine erythrocytes to give hemadsorption plaques.80

In certain kinds of cell cultures a CPE. with the appearance of ameboid cells and sloughing, is produced. Primary cultures of human amnion were used by Weller and Neva, the CPE being apparent in five to six weeks. Rubella virus grows to high titer in primary cultures of rabbit embryo cells with the appearance of CPE in six to eight days. 102 Other investigators have used a continuous line of rabbit kidney cell cultures (RK-13) which, with adaptation of the virus, show CPE in about four days, and still others have used a continuous line of monkey kidney cells. When CPE is produced in a reasonable time it allows a practical direct

method of titrating neutralizing antibody. Much of the data presently available on neutralizing antibody have been obtained by the interference type of test, or directly in RK-13 cell cultures: the latter gives somewhat lower titers than the former.

Virus so cultured appears, on the basis of filtration experiments, to be 100 to 300 m μ in diameter, to be of the RNA type, and to generally resemble the myxoviruses. There seems to be only one serological type.

Many attempts have been made to reproduce the disease in experimental animals without success. With the availability of culture virus, it has become clear that subhuman primates and nonprimate conventional experimental animals respond immunologically, often with the production of high titer neutralizing antibody, in the absence of clinical signs of disease. Of the latter, the ferret appears to be one of the most sensitive experimental animals. For the present, studies on the pathogenesis of the disease and evaluation of prophylactic and therapeutic measures continue to depend upon the use of human volunteers.

Pathogenicity for man. As indicated above, rubella is a relatively common childhood disease. With the availability of methods of detecting the virus and the titration of neutralizing antibody, studies on human volunteers infected intranasally with 100 $TCID_{50}$ of virus have shown (a) that the presence of neutralizing antibody prevents the clinical disease; (b) that virus is found in the serum and urine nearly a week before the onset of symptoms, disappearing soon after the appearance of rash and coincident with rise in antibody titer; (c) that virus is recoverable from the nasopharynx in greatest amount coincident with rash and continues to be shed from this area for 10 days to two weeks after the appearance of rash; and (d) that antibody titer continues to rise as long as four weeks after the onset of symptoms. Immunes, i.e., those showing pre-existing neutralizing antibody, not only show no symptoms, but apparently do not shed virus, nor does antibody titer rise.

Immunity is acquired by clinical or inapparent infection, and the proportion of persons showing neutralizing antibody rises with age. Immunizing infection appears to occur through elementary and high school, some 20 per cent of preschool children showing neutralizing antibody, with the proportion rising to 80 per cent in the 17- to 20-year age group. A survey made prior to the 1964–1965 epidemic prevalence of the disease showed that the proportion of immunes increases from about 75 per cent in the 14- to 19-year age group to 89 per cent in the 31- to 44-year age group. The agespecific attack rates observed during the 1964–1965 period were consistent with the assumption that neutralizing antibody is associated with effective immunity to the disease.

The immune status of women in the childbearing age group, 14 to 44, is of special significance because of the teratogenic effects of the infection on the fetus noted above. Several serological surveys have indicated that the overall percentage of susceptibles in this group in cities in the United States is about 17 to 18, and in Hawaii the susceptibility rate was 48 per cent. Canadian studies⁵³ have shown also that antibody titers tend to decline between 25 and 40 years of age. A study of a group of 65 student nurses, aged 18 to 23, exposed to infection and observed over a 12-week period, showed that 17 per cent initially lacked neutralizing antibody, and of this portion 55 per cent developed rubella, about half in clinical form and the other half as inapparent infection demonstrated by virus isolation and the appearance of neutralizing antibody. 112 Much higher attack rates in nonimmunes, nearly 100 per cent, have been observed in military recruits.

Intrauterine infection of the fetus apparently occurs readily in consequence of clinical or inapparent infection as demonstrated by the presence of the virus in aborted fetuses, stillborn infants, and infants with or without abnormalities. In fact, as much as 75 per cent of infected infants were born of mothers with no history of clinical rubella. Infected infants serve to disseminate the infection, often for several months, in spite of the presence of neutralizing antibody.

The teratogenic mechanisms resulting in associated defects remain speculative. The virus may be widely disseminated in the fetus; in fact virus carrier cell cultures have been obtained from congenitally infected infants, ¹⁰¹ and it has been suggested ⁸⁹ that viral proliferation interferes with normal cell growth *in utero*, and possibly subsequently also. The concurrent presence of antibody and virus has been taken to suggest that antigen-antibody reactions, as well as direct effects of the virus, may contribute to the observed consequences of fetal infection. ¹⁶

The isolation and culture of the virus makes possible the development of an effective vaccine, killed or in living attenuated form, but such an immunizing agent is not yet available.

The Rabies Virus¹⁰⁵

The rabies virus, together with poliovirus, is the most highly neurotropic of the viruses. All mammals, and some birds, are susceptible to infection, and in most species, but with important exceptions, it is practically uniformly fatal. The disease, also known as hydrophobia, has been known from ancient times in the Old World in Egypt, Greece, Italy, and other parts of Europe, and the evidence suggests that it was imported into the Western Hemisphere from Europe. Prior to the eighteenth century, it was primarily a disease of wild animals, but in modern times the dog has assumed increasing importance and is responsible for the majority of cases of human infection with the rabies virus. The infectivity of saliva was demonstrated early in the nineteenth century; the virus was studied by Pasteur in the 1880's with the

development of prophylactic treatment and in 1903 was shown by Remlinger to pass bacteria-proof filters.

Morphology and cell culture. Until relatively recently the relation of the rabies virus to other viruses has been obscure. While it is set apart by its marked neurotropism and antigenic individuality, it appears to belong to the myxovirus group. It is an ether-sensitive RNA virus showing helical symmetry and resembling the influenza rather than the paramyxovirus group in its helical diameter of 9 m μ . The virus particle size has been found to range from 160 to 200 m μ by filtration studies, and 110 to 130 $m\mu$ in electron micrographs of negatively stained preparations,5 with a single or double limiting membrane. Projections similar to those found in other myxoviruses have been described.³⁴ Filamentous particles have also been observed which are considered to be unravelling double helices.⁹⁹

While the rabies virus appears to be homogeneous antigenically, a number of antigenic components are demonstrable, ⁸³ and small immunological differences between the usual canine strains and the virus causing paralytic disease in cattle and transmitted by bats are demonstrable by the neutralization test. There are also strain differences in ability to invade the salivary glands.

The virus may be grown in embryonated eggs. Without adaptation, growth in the chick embryo is sufficiently slow that it is outstripped by the growth of the embryo and yields of virus are poor. It may be grown to greater yields in the more slowly developing duck egg, and duck egg virus (DEV) is used as an immunizing agent (see below). The virus may also be grown in mouse brain tissue culture, and in cultures of some nonnervous tissues, viz., newborn hamster kidney fibroblasts, rabbit endothelium, and the WI-38 strain of human diploid cells, but without CPE. In such cell cultures the virus is demonstrable by the fluorescent antibody technique.73 On continued maintenance on hamster fibroblasts or human diploid cells, virus accumulates in the progeny of infected cells in sufficiently large masses that mitosis is inhibited with eventual cell lysis. 45

Pathogenicity.35 The neurotropism of the rabies virus is evident in the pathogenesis of the disease. From the site of the local injury where it is introduced into the tissues, the virus passes along the nerves to the central nervous system; viremia is uncommon, but hematogenous spread may occur under experimental conditions such as infection of the dog by the intravenous route. The significance of the neural pathway is indicated by the experimental infection of nerves, producing infection first in the connected part of the spinal cord, and by the prevention of spread by resection of the nerve, the resected portion remaining infective. There is also evidence that, following its introduction directly into the brain, the virus may spread centrifugally, appearing in nerves such as the brachial and sciatic, producing interstitial neuritis. It has been suggested that the virus reaches the salivary glands in a similar manner.

Infection results in extensive destruction in the cerebral and cerebellar cortices, the midbrain, basal ganglia, pons, and medulla, with neuronal degeneration and demyelination. Similar changes are observed in the spinal cord and are most marked in the posterior horns. There is general hyperemia and mononuclear infiltration, and there may be small perivascular hemorrhages. The extent of these changes is related to the duration of the disease, and when death occurs soon, they may be minimal.

Negri bodies. The acidophilic inclusion body, the Negri body, is pathognomonic of rabies. It is found within the cytoplasm of the large ganglion cells, most abundantly in Ammon's horn, in the pyramidal cell layer of the cerebral cortex and the Purkinje cell layer of the cerebellum, in the cranial nerve nuclei and in the basal ganglia. They are round in shape when lying free in the cytoplasm, may be compressed to an oval form by the nucleus, and are elongated when present in the dendrons. There is a considerable range in size of the Negri body, commonly given as 2 to $10~\mu$, but both smaller and larger bodies may be found.

When stained by Giemsa, the Negri body consists of a light blue ground substance containing red to red-violet basophilic granules. Sellers' stain, basic fuchsin and methylene blue in methanol, is regarded as superior by some workers; it stains the Negri body bright red and the internal granules are blue. The Negri body is stained

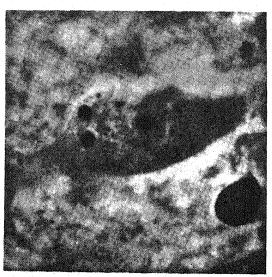


Figure 279. Negri bodies in a nerve cell in a section of the brain of a rabid cat. The three dark spherical bodies within the cytoplasm of the cell are Negri bodies. (Schleifstein.)

specifically by the fluorescent antibody technique which also stains the small eosinophilic inclusion bodies which are not ordinarily considered to be specific.⁵⁴

Negri bodies are not invariably present in infections with canine, or street, virus and cannot be demonstrated in approximately 10 per cent, at times as much as 25 per cent, of infections even though the virus may be isolated. They are not produced, at least not in what is considered to be characteristic size, in infections with the rabbit-adapted, or fixed, virus.

Infection in lower animals. As indicated above, rabies virus occurs in reservoirs of infection in lower animals, most often carnivorous mammals. While practically any mammal, including herbivorous animals, may be infected at one time or another, the kinds of animals providing the reservoir of infection differ from one part of the world to another. The infection is endemic in foxes in Western Europe, and in the wolf in Eastern Europe, and may assume epizootic form from time to time in these animals. In West Africa dogs, both wild and domestic, are commonly infected, and the canine disease is called oulou fato locally, but the epidemiological behavior of the disease suggests that the canine infection is secondary to a reservoir of infection in some small rodent. In South Africa the infection is endemic in small carnivora, the meerkat and related animals, and the mongoose may contribute significantly to maintenance of the infection. In India jackals and wild dogs, and probably the mongoose to a lesser extent, are the important mammalian hosts of the virus.

In North America, Canada is relatively free from rabies,⁴³ but in the southern part of the continent the disease is endemic, and occasionally gives rise to epizootics in foxes in the eastern and southeastern part of the United States, in skunks and other small rodents such as squirrels, raccoons, opossums, and muskrats in the Middle West and West, in coyotes in the West. Larger animals, such as deer may be infected also. In addition to these reservoirs of infection, rabies is also present in bats (see below).

The infection in wild animals may be regarded as the natural form of the disease. It serves as the source of infection for the domestic dog to give an urban type of the disease, the form most important to man.

Rabies in dogs. The infection in dogs is

closely similar to that in wild animals and also to the human disease, and may be considered as representative. Dogs are infected by wild animals, or by other dogs, by the bite of the infected animal. Disease is not inevitable, and an appreciable portion of dogs bitten by a rabid animal, more than half, do not develop the disease. The usual incubation period is three to 10 weeks, but may be as short as 10 days, and under experimental conditions is dependent upon the amount of virus inoculated. The virus is present in the saliva prior to the appearance of characteristic clinical signs of the disease, and the animal is infectious during the last three to five days of the incubation period.

The disease takes two forms, the one furious or mad rabies and the other dumb rabies, depending upon the prolongation of the excitation period. At first the animal becomes restless and apprehensive and may become apathetic or seek out companionship. It then becomes hyperexcitable, responding to minimal stimuli, and runs about snapping and biting without discrimination, apparently oblivious of its surroundings. Beginning paralysis results in drooling of saliva due to inability to swallow, the gait may become abnormal, and the vocal cords are affected to give an eerie quality to the bark or howl. In dumb rabies the phase of excitation is relatively short, the animal appears to be profoundly depressed and unaware of its surroundings, overt paralysis becomes increasingly evident, and in general the more prolonged the disease the greater the paralysis. In either case the disease is practically invariably fatal within 10 days after onset of symptoms, although rare instances of recovery from the paralytic disease have been described. Occasionally death from rabies may occur suddenly without overt symptoms of disease.

On autopsy virus is present in the brain and cord, in the salivary and lacrimal glands, and in the kidney and pancreas, but seldom in the blood, and in general is not found in tissue of mesodermal origin. As noted earlier, strains of virus differ somewhat in their tissue affinities, some strains infecting and multiplying in the salivary glands to a greater extent than others.

The clinical features of the disease are characteristic, and the canine infection is diagnosed by the presence of Negri bodies, but not eliminated by their absence, usually in impression smears of tissue from Am-

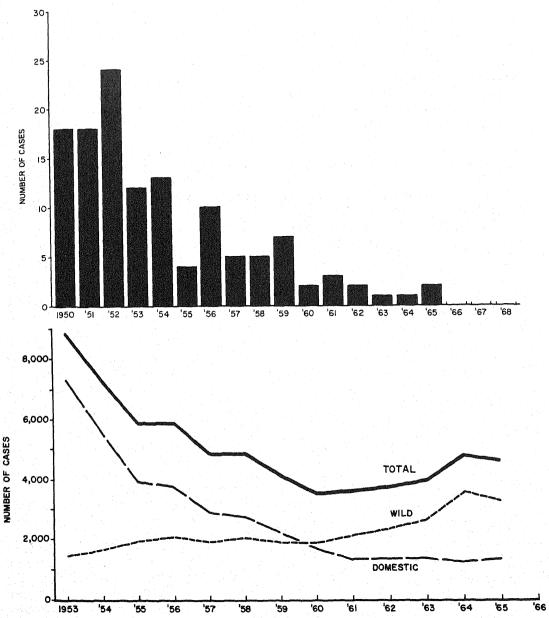


Figure 280. The incidence of rabies in the United States as shown (upper) by reported human cases in the period 1950-1965 and (lower) by reported cases in animals in the period 1953-1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare.)

mon's horn and/or by isolation of the virus, commonly by intracerebral inoculation of mice. The diagnosis of canine rabies is of practical significance in relation to the institution of prophylactic inoculation in man exposed to infection by the bite of a dog.

Rabies in bats.³⁹ The occurrence of bat rabies, or chiropteran rabies, was first recognized in Brazil in 1908. The infection has

since been found to be widely distributed in vampire bats, of which the most important species in this connection is *Desmodus rotundus murinus*, not only in South America, but also in Central America and Mexico. Since 1953 the infection has been found in a number of species of nonhemophagous bats in various parts of the United States.

The virus found in bats, and in animals

infected by bats, differs from the canine strains in that it produces an ascending myelitis to give a spreading type of paralysis which is almost invariably fatal in animals other than bats. In cattle the hind limbs are paralyzed first, and the disease in man has at times been thought to be an infection with an aberrant type of poliovirus.

While the furious type of rabies is seen in both naturally and experimentally infected bats and is frequently fatal, infectious bats may survive for extended periods, as long as three months, and appreciable numbers survive to become healthy carriers of the virus. Bats are, therefore, an exception to the general rule that the infection is almost invariably fatal. The role of the brown fat in the bat infection has been emphasized by Sulkin and his co-workers. The virus has been isolated from this tissue taken from naturally infected bats, and it has been found that the virus does in fact persist in this tissue, sometimes referred to as the hibernation gland, which functions as a "reservoiring mechanism" where little or no multiplication occurs during hibernation, but on warming the virus multiplies to reach detectable levels in other tissues.4

The vampire bat bites at night to transmit the infection to cattle and other domestic animals and to man. The fatal paralytic disease in cattle has frequently assumed epizootic proportions and is known locally as mal de caderas in Brazil, rabia parisiante in Argentina, and renguera, tronchado, or derriengue in Central America and Mexico. Within affected areas the disease has occurred in 20 to as much as 50 per cent of cattle and other domestic animals. During 1929 to 1931 in Trinidad over 1000 cases occurred each year, and there were 13 cases of fatal human disease in 1929 and four the following year.

Nonhemophagous insectivorous and fruiteating bats in the United States have been found infected in 15 states, ranging from a number of southern states to areas as distant as Montana, Minnesota, Ohio, New York, Delaware, and New England. Eabies virus has been isolated from species of Dasypterus, Lasiurus, Myotis, Tadarida, Pipistrellus, Eptesicus, and Chilonycteris. Of these, the Mexican free-tailed bat, Tadarida mexicana, appears to be relatively heavily infected in Texas, as indicated by an incidence of neutralizing antibody in 16 to 70 per cent, varying with location, of speci-

mens examined. In a detailed study carried out in Florida,⁴⁰ a total of 5503 colonial and free-living bats were examined, and the incidence of infection was found to be 0.2 per cent in the colonial forms and 1.5 per cent in the free-living forms. There appears to be little doubt that the bats constitute a significant reservoir of rabies infection in the Western Hemisphere.

Attenuation. Alteration of the virulence of rabies virus by serial animal passage is the classic example of attenuation of virulence of microorganisms by this method and was applied by Pasteur in his development of attenuated virus for prophylactic immunization. The canine virus, as encountered in nature, was designated by him "street virus." On intracerebral inoculation into the rabbit, disease is produced after an incubation period of about two weeks, but as the infection is passed serially in this animal, the incubation period is progressively reduced, reaching eight days after 20 to 25 passages, and a limit of seven days after another 20 to 25 passages. This modified virus was called "fixed virus" by Pasteur.

Fixed virus is characterized by an enhanced neurotropism, an inability to produce Negri bodies in the infected animal, and a reduced ability to invade the salivary glands. Pasteur's original strain of fixed virus, together with many of its substrains, is still available and has gone through 2000 or more passages in the rabbit. These strains vary among themselves in their virulence, as indicated by differences in incubation period and ability to produce infection by peripheral routes of inoculation in various experimental animals, but in general neurotropism is sufficiently enhanced that they are avirulent for the rabbit, dog, and man by the subcutaneous route. Fixed virus may also be prepared in other animals such as the mouse, guinea pig, or monkey by serial intracerebral passage; on intracerebral passage in the mouse, for example, street virus loses its ability to produce Negri bodies after about 25 passages.

The virus may also be adapted to the chick and chick embryo by continuous passage. The best-known of these strains is the strain isolated by Johnson in 1939 from a human case in day-old chicks and known as the Flury (the patient's name) strain. It was passed 136 times in chicks without observed changes except a decrease in the incubation period from 30 to six days, and

then was passed in chick embryos. After repeated passage in the embryo, its pathogenicity for rabbits and dogs by extraneural routes of inoculation had disappeared, and at the one hundred and seventy-fourth embryo passage intracerebral pathogenicity for the adult mouse was lost, and both dogs and rabbits are resistant to intracerebral inoculation with this high egg passage, or HEP, strain.⁷⁷ Such attenuated strains are of particular interest as immunizing agents.

The disease in man. Human infection with rabies virus is acquired by the bite of an infected animal. The infectiousness of the animal is determined by the presence of virus in the saliva, and it may be present for a few days, probably not more than five, prior to the onset of symptoms. This time element is significant for if the animal does not develop rabies within 10 days, the saliva was probably not infectious at the time of the bite. The incubation period of the human disease ranges from a minimum of two weeks, or possibly slightly less, to as long as five to seven months and is related to the severity of the bite and the location and innervation of the muscle tissue involved. Not all persons bitten by a known rabid animal develop the disease. Whether or not infection occurs is influenced by factors such as the severity of the wound, the removal of saliva when the bite is through heavy clothing, etc., but among equally severely bitten persons less than half may develop the disease. There is evidence that the infection may be acquired by other routes, possibly by inhalation, in caves frequented by bats,³⁰ and an association between such caves and the occurrence of rabies in foxes has been observed.48

The clinical disease in man is similar to that in the dog. The prodromal symptoms include fever, headache, malaise, nausea, and anorexia, and there is anxiety or depression, and often sensations of itching, burning, or tingling at the site of the wound. The onset of the excitation phase is gradual and indicated by increasing nervousness and apprehension. The characteristic symptom is the painful spasmodic contraction of the muscles involved in swallowing and the accessory muscles of respiration when fluid comes in contract with the fauces, and subsequently suggestion of the act of swallowing may precipitate muscle spasm; this feature of the disease gives it the name hydrophobia. As the disease progresses

there may be muscular contractions and tremor, choking, and resulting cyanosis; convulsions occur, and there is often maniacal behavior. Such periods of excitation may be interspersed with lucid intervals. Death usually occurs during convulsions. In some instances the patient survives the period of excitement and becomes apathetic, stupor and coma follow, and an ascending type of paralysis develops. So far as is known the disease in man is invariably fatal.

The pathological changes in human rabies are similar to those seen in dogs and include some degree of hyperemia and severe neuronal degeneration in the midbrain, basal ganglia, and pons. Degenerative changes are marked in the nuclei of cranial nerves and in the thalamus and hypothalamus, and Negri bodies are present. Neuronal degeneration in the cord is most marked in the posterior horns, and when the bite has occurred on an extremity, the corresponding posterior horn shows extensive destruction with neuronophagia and may extend into the dorsal root ganglia.

Laboratory diagnosis. 106 The laboratory diagnosis of rabies is usually of primary importance in the animal which has bitten or otherwise exposed man. As indicated above, the saliva is infectious prior to the development of symptoms. Premature destruction of the animal is undesirable and if possible the disease should be allowed to develop if present to facilitate diagnosis. Such diagnosis, in lower animals or man, is based on the demonstration of Negri bodies, usually in impression smears of Ammon's horn. Giemsa or Sellers' stain may be used, and the fluorescent antibody technique allows the identification of smaller bodies not ordinarily identifiable as Negri bodies.⁷⁹ The technique may, in fact, be used to identify the virus in the salivary glands of infected animals.55 The virus may also isolated by intracerebral inoculation of mice and demonstration of Negri bodies in the mouse brain.

Immunity. Complement-fixing and neutralizing antibodies are formed in response to immunization with vaccine and in unimmunized individuals during the course of the disease. The immune response to infection is not an effective one in man, nor does antibody activity have diagnostic value except in animals such as bats which recover from the disease. Antibody titrations are useful, however, for assaying the response to

immunizing preparations, identification of virus by neutralization tests, and other experimental purposes.

Prophylaxis. There seems to be no significant change in the antigenicity of rabies virus on adaptation to give fixed virus, and effective immunization to rabies in man and dogs is possible because of the long incubation period in the first instance, which allows sufficient time to induce an effective immune response. Immunization of dogs on a mass basis makes possible the control of the urban type of the disease. The immune response to vaccines is not long lasting, and in the case of dogs must be repeated each year.

The extended incubation period in rabies makes possible the development of an effective active immunity even though the immunization is begun after exposure. Pasteur's original method was the inoculation of the exposed person daily with emulsions of fixed virus in rabbit spinal cord, beginning with cord which had been dried 14 days, and gradually increasing the amount of virus by the use of cord dried for shorter and shorter periods. Subsequently other methods, using gradually increasing doses of active virus or virus inactivated by various chemical and physical methods, have been used. In this country fixed virus in rabbit brain tissue, inactivated by phenol (Semple vaccine), formalin, etc., is given in daily subcutaneous inoculations for a period of 14 or 21 days, and similar preparations are used for the immunization of dogs. Phenolized vaccines may be stabilized by lyophilization and still retain immunogenic potency. All such vaccines contain relatively large amounts of nerve tissue, and there is reason to believe that complications, usually neuritis or myelitis with occasional permanent damage, are in large part the consequence of an iso-immune response to organ-specific antigen (Chap. Thirteen).

Vaccines prepared from egg- or cellcultured virus, and consequently lacking contaminating nerve tissue antigen in large amount, have been of considerable interest. The chick embryo-adapted HEP Flury strain¹¹⁰ noted above is effective for primary immunization of domestic animals, but not of man or subhuman primates, although it may be used for booster inoculation in the maintenance of pre-exposure immunity in high risk personnel.⁴⁴ Vaccine prepared from the HEP Flury strain grown in human diploid cell culture (WI-38) has been found to have greater antigenicity in monkeys, 136 but has not been tested in man. Vaccine prepared from virus cultured in duck eggs, and inactivated with β -propriolactone, is a more effective immunizing agent 6,114 and is used for human immunization, but none of these vaccines equal vaccines of the Semple type in immunogenic potency. Assay of potency is carried out in the United States by the Habel method, the protection of actively immunized mice against 1000 LD_{50} of virus by the intracerebral route. 36

Antiserum contains neutralizing antibody demonstrable experimentally and has been of interest as a prophylactic or an adjunct prophylactic for the human disease. It is evident that if antiserum containing neutralizing antibody is given very soon, within a few days of exposure to infection, it gives an appreciable protective effect, and a combination of antiserum and vaccine has been found to give better protection than either alone. 11, 57, 113

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Chapter Thirty-nine

THE PICORNAVIRUSES
(POLIOVIRUSES, COXSACKIE
VIRUSES, ECHO VIRUSES, EMC
VIRUSES, COMMON COLD
VIRUSES); THE HEPATITIS VIRUSES;
THE ADENOVIRUSES

The descriptive term picornavirus covers a group of small (pico) RNA viruses which resemble one another closely in morphology and cytopathic effect (CPE) produced in cell cultures. The term nanivirus (nanus, dwarf) had been proposed for this group, but did not gain general acceptance and has been discarded. The viruses of this group fall into two main subgroups on the basis of the pathogenesis of the diseases they produce. 138 One is the enterovirus group,7, 183, which includes the polioviruses, the Coxsackie viruses, and the ECHO viruses, 113 together with enteroviruses found in lower animals. The other is a large group of antigenically diverse viruses which are etiologically related to the common cold and which have been called rhinoviruses, coryzaviruses, "respiratory" viruses, etc. While the general outlines of this classification, and further subdivision, are apparent, details and matters of nomenclature remain somewhat fluid. 155 The occurrence of intermediate types has tended to blur distinctions, but the proposal that the viruses making up the enterovirus subgroup be designated arbitrarily by number¹²⁰ has not been adopted.

These viruses are 20 to 30 m μ in diameter and ether-resistant, and the contained RNA appears to be primarily single-stranded.

Those which have been adequately studied have cubic icosahedral symmetry and 32 capsomeres; the particle is probably in the form of a rhombic triacontahedron, and there is no enveloping membrane. They grow in a variety of cell cultures, with some strain or type differences, and are found in cytoplasm as masses of orderly arrays of virus particles to give inclusion bodies with a crystalline-like appearance.⁹⁸

The hepatitis viruses are very poorly known, and it is by no means clear that they are related in any way to the picornaviruses. The virus of infectious hepatitis is present in the feces and infection is contracted through the ingestion of contaminated water, so it would appear to be an enteric virus in this sense, though not an enterovirus as the term has been used. The virus of serum hepatitis apparently occurs only in the blood, and its relation, if any, to that of infectious hepatitis, or of either of these to the hepatitis viruses of lower animals, is quite unknown.

The adenoviruses are set sharply apart from the picornaviruses in that they are DNA viruses and generally somewhat larger in size, but these viruses are considered here because of the association of many of them with upper respiratory disease.

The Polioviruses 51, 118

Poliomyelitis (infantile paralysis, acute anterior poliomyelitis, Heine-Medin disease) was differentiated as a clinical entity by Heine in 1840, and described in epidemic form by Medin in 1891. The viral nature of the etiological agent was demonstrated in 1908, when Landsteiner and Popper transmitted the disease to monkeys.

The infection is widely distributed throughout most of the world, and the occurrence of the disease in young children for the most part is, as in many other diseases of childhood, an expression of a high probability of contact with the polioviruses. It is estimated that in Chicago most children have been infected once within the first four years of life, and by the sixth birthday 75 per cent have been infected twice. The age at which primary infection occurs is subject to some variation and is affected by social and economic factors (see below).

The great majority of infections are symptomless, and the disease, paralytic or nonparalytic, appears to be the exception. With the waning of passive immunity of maternal origin, repeated infection, with or without disease, results in an immunity, and most adult serums contain neutralizing antibody which is invariably present in pooled materials such as y-globulin concentrates. The incidence of the disease is similar to that in certain other infections, such as meningococcal infection, in which the causative agent is widely distributed in the human population, but the infection is usually symptomless, and the disease tends to appear in sporadic form or occasionally in epidemics.

Poliovirus. ¹⁷⁴ These are among the smallest of the viruses and estimations of size of the spherical particle, based on differential filtration, sedimentation, and direct observation in electron micrographs, range from 22 to 32 m μ in diameter. The virus particles may occur in orderly arrays and have been prepared in the form of bipyramidal crystals (Chap. Three).

The activity of the virus is stable over a relatively wide pH range, pH 4 to 10, over periods of several days at refrigerator temperature; it is resistant to treatment with glycerol, ether, and 1 per cent phenol but is labile to oxidizing agents and ultraviolet irradiation. It is inactivated in 30 minutes at 50 to 55° C. and destroyed in milk by pas-

teurization. It persists in feces and sewage at refrigerator temperatures for extended periods, for as long as three months, and in water has been reported to be somewhat more resistant to chlorination than the vegetative cells of many bacteria. Its inactivation by formalin has been of interest, since this substance is used in the preparation of inactivated vaccines, and prolonged treatment results in the disappearance of antigenicity. The rate of inactivation is exponential over a considerable range (Chap. Six), but treatment based on extrapolation of this rate has resulted in incomplete inactivation.¹⁷³

Antigenic types. Strains of poliovirus are not necessarily immunologically identical, and these viruses have been separated into three types by the specificity of neutralizing antibody. These types are numbered, and are also designated by the name of the prototype strain, e.g., type 1 or Brunhilde, type 2 or Lansing, and type 3 or Leon. Minor antigenic differences occur between strains of all three types. 54, 196 Of these, type 1 is found most often, but more than one type may occur in a single epidemic. Typing is carried out by cross-neutralization tests, at first with monkeys, but now largely in tissue culture. Of a group of 100 strains of global origin typed in the monkey, 85 were found to be type 1, 12 type 2, and three type 3; of a group of 274 strains isolated in this country and typed in tissue culture, 204 were type 1, 22 were type 2, and 48 were type 3. The distribution of the types appears to be world-wide, but it is uncertain as to whether figures such as these reflect the relative prevalence of the three types in the human population.¹⁷⁷ These types differ somewhat in other ways. For example, the Lansing strain is one of the more readily adaptable to rodents, and the Leon type has relatively low virulence for the monkey. There are also differences in virulence between strains. the Brunhilde strain being one of the more virulent strains of type 1.

Two kinds of complement-fixing antigen are present in the virus which are separable by density gradient centrifugation, fraction C and fraction D, with infectivity associated largely with the latter, heavier, fraction. These antigens are separable by gel-diffusion, and D is heat-labile while C increases in amount on heating, presumably as a

VARIATION 915

result of a D \rightarrow C conversion rather than an unmasking of C.²² The occurrence of these two kinds of antigens resolves to a considerable extent anomalous results previously obtained with the complement-fixation reaction. Some workers have introduced the designation N for infectious antigen and H for heat-stable antigen, *i.e.*, C = H and D = N, since it is not established that all particles which are antigenically C invariably sediment in the C zone characteristic of empty shells.⁹⁰

Tissue culture. Beginning in 1949, poliovirus has been grown in cultures of a variety of tissues, including human embryonic tissues, human foreskin and testis, monkey kidney and testis, and in HeLa cell culture, in the Maitland-type flask culture, and in roller and static tube culture. By appropriate adjustment and timing of inoculums, interference among the three immunological types is demonstrable.

A degenerative type of cytopathology is produced which is clearly apparent in the fibroblastic outgrowth as a progressive granulation and eventual destruction.⁸⁴ The CPE is more pronounced with some virus strains than others; e.g., The Lansing strain is feebly cytopathogenic while the Y-SK strain, also type 2, produces extensive degeneration. Further, pathogenicity in tissue culture and pathogenicity for experimental animals are not necessarily parallel.

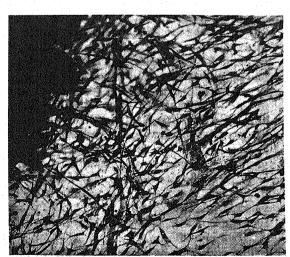
The cytopathic effect is specifically neu-

tralized by antiserum, and thus providing the basis for tissue culture typing of virus strains and may be quantitated for virus assay by the plaque method. ⁴¹ There is evidence that the observed cytopathology is in part a toxic effect of some strains of poliovirus in which the toxicity and infectivity are immunologically independent. ¹

Poliovirus may also be grown in tissue culture by the monolayer technique in which the layer of cells is inoculated, incubated for 45 minutes, and, after the excess inoculum is removed, overlaid with agar. Discrete foci of degeneration, or plaques, occur when the inoculum is sufficiently dilute and may yield differentiable virus strains on subculture.

Variation. Serial passage in tissue culture may result in changes in virulence for experimental animals. The Lansing strain, carried in human embryonic tissue culture, for example, showed a decrease in mouse virulence without a corresponding decrease in virulence for the monkey or alteration in tissue culture infectivity, and the Brunhilde strain, carried similarly, showed a marked decrease in virulence for the monkey. In the same way, after passage in monkey testis tissue culture, the Lansing strain lost its pathogenicity for the mouse but retained its virulence for the monkey, but adaptation to rodent hosts is associated with loss of virulence for the monkey.

Variation in virulence for the mammalian



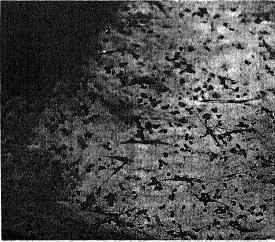


Figure 281. Preparations from roller-tube cultures of human embryonic skin-muscle tissue seven days after cultures were initiated. Left, uninoculated culture; right, inoculated three days previously with the Brunhilde strain of poliomyelitis virus. Few cells remain in the infected culture, the majority having been destroyed by the cytopathogenic action of the virus. Hematoxylin and eosin. (Weller: New Eng. J. Med.)

host may be accompanied by changes in plaque morphology, as in the case of the *d* variant. ^{55, 193} Variants of reduced virulence but with, or perhaps because of, abnormal capacity for growth in tissue culture have been produced by rapid tissue passage with large inoculums by Sabin and his associates. ¹⁶⁹ These have been of special interest as immunizing agents (see below)

The disease in man. So far as is known. natural infection with poliovirus occurs only in man. The virus is demonstrable in the pharyngeal secretions and stools of infected individuals, including contacts who do not develop the disease, and there appears to be a large reservoir of infection in the human population. The infection is spread by contact, though in precisely what way is uncertain, and cases of the disease are distributed radially from an original focus of infection. The incubation period is variable, and is usually given as one to two weeks, although it may be as long as four or five weeks. The clinical disease is separable into three types: the abortive, the nonparalytic, and the paralytic.

The initial symptoms of the disease are those of a mild upper respiratory infection with nonexudative pharyngitis and headache, or of gastroenteritis with nausea and vomiting, but constipation rather than diarrhea, and in either case there is a febrile reaction. When the disease does not develop beyond this point, it is known as abortive poliomyelitis, but it cannot be diagnosed as such without isolation of the virus or demonstration of an increase in antibody titer between paired serums.

The disease may progress with the development of symptoms of involvement of the central nervous system, including pain and stiffness of the muscles of the neck and back and Kernig's sign. This is nonparalytic poliomyelitis. In paralytic poliomyelitis a flaccid paralysis and spasm of unparalyzed or partially paralyzed muscles occur at the terminal phase of the febrile period, progressing for only a short time, usually not beyond 24 hours. Virus disappears rapidly from the spinal cord but may continue to be eliminated in the feces for some time.

The lesions in paralytic poliomyelitis consist of pathological changes in neurons, ranging from mild chromatolysis to complete destruction, and are characterized by local perivascular infiltration with lymphocytes and macrophages, with phagocytosis of the necrotic motor cells. The anterior horn of

the spinal cord is involved, and the destruction of the large nerve cells giving rise to the motor fibers of the peripheral nerves results in the flaccid paralysis. The selective distribution of lesions in the brain is distinctive, differentiating poliomyelitis from the viral encephalitides, in which there is a more or less general diffusion of the virus into the brain. In poliomyelitis the lesions in the cerebral cortex are confined to the motor and premotor areas, rarely extending into the postcentral cortex, and the visual, auditory, and association areas are not involved. There is no impairment of mental faculties, and symptoms of encephalitis disappear when the acute stage of the disease subsides. In the hindbrain the motor cells of the medulla and pons, the vestibular nuclei, and related centers in the cerebellum may be involved, but the neocerebellar complex is not affected. Bulbar poliomyelitis is a consequence of a concentration of neuron destruction in the parts of the medulla containing the motor nuclei of the cranial nerves, with effects ranging from weakness of facial muscles to involvement of the respiratory and vasomotor centers.

Pathogenesis. The pathogenesis of poliomyelitis has been worked out in detail in the chimpanzee and, since this experimental infection appears to be a counterpart of human infection, may be assumed to represent, in a general way at least, the pathogenesis of the human disease. According to Bodian, 17, 19 within a few days after the ingestion of an infecting dose high concentrations of virus are demonstrable in the lymphoid tissues in the tonsils and in Peyer's patches in the intestine. Multiplication of the virus and its excretion from these areas account for its appearance in throat swabs and washings and in the feces. The deeper lymph nodes, cervical and mesenteric, are infected by drainage from these primary sites, and the virus invades the blood stream by way of the lymphatic vessels and the thoracic duct to produce viremia. The viremia is seldom demonstrable in man, possibly because it occurs very early. The infection is spread via the blood stream to various susceptible tissues, including other lymphatic tissue, brown fat, and the central nervous system.

The pathway of invasion of the central nervous system is somewhat uncertain. It may be by way of the blood, but there is also evidence of centripetal spread of the virus along the axons of peripheral nerves, and

Sabin¹⁶⁶ regards the regional nerve ganglia and their connections with the central nervous system as playing a significant role in the pathogenesis of the disease. Virus and lesions are not present in the olfactory bulbs, and virus occurs only rarely in human celiac ganglia, but the vagus and visceral afferent fibers represent a possible pathway from the gastrointestinal tract to the central nervous system. It is also possible that lymphatic tissue is not the exclusive site of multiplication of the virus, but other extraneural tissues may also serve as foci of infection.

The virus is present in the feces and laboratory diagnosis is dependent upon its isolation and serological identification. Monkey kidney, HeLa, KB, and human amnion cell cultures appear to be about equally sensitive for isolation.³¹ Serological identification consists of neutralization in tissue culture with known antiserum¹³² and the fluorescent antibody technique has been applied also.⁷⁶

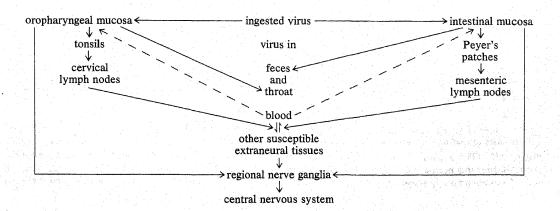
Predisposing factors. In the abortive type of disease, the infection progresses no further than the alimentary or viremic phase, a limited invasion of the central nervous system with reparable damage occurs in nonparalytic poliomyelitis, and extensive invasion and destruction within the central nervous system, which may be largely spinal, or bulbar and bulbospinal, occur in the paralytic form. The factors which determine whether the virus is to enter and multiply within the central nervous system are known in only a limited way.

There is no doubt that specific immunity plays an important part in limiting spread of the infection, but certain nonspecific factors are significant also. Evidence from various sources indicates that injections for immunization of children against diphtheria, pertussis, and tetanus may influence to some degree the site of paralysis in cases occurring up to approximately three weeks following the inoculation, but it is not clear that the incidence of paralysis is affected. 103 It is also established that heavy physical exercise during the prodromal stage of the disease results in more severe paralysis, and paralysis is more likely to occur during pregnancy. An increased incidence of the bulbar form of the disease occurs following tonsillectomy or adenoidectomy. The surgical trauma may open the way for virus already present in the pharynx to gain access to the cranial nerves by which it may reach the medulla directly, or local changes may occur in the permeability of the vascular system supplying the neuraxis. 18, 126 In any case, elective operations in the oropharyngeal area, including dental extractions, should not be performed during epidemics of poliomyelitis, and preferably not during the late summer and early fall, when the prevalence of the infection is high.

There is also no doubt that age is highly significant in the severity of the disease. It is less severe in young children than adults as indicated by an increase in both disability and case fatality with increasing age. The incidence of the more severe bulbar type of infection is appreciably greater in the higher age groups, and the case fatality rate is two to five times as great in adults as in children under five to seven years of age.

Epidemiology. The influence of social

Pathogenesis of Poliomyelitis (after Sabin)



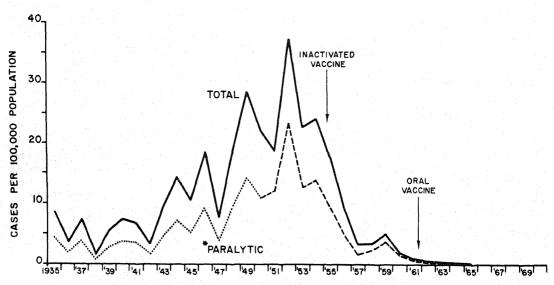
and economic conditions on the epidemiological character of infectious disease has been discussed elsewhere (Chap. Ten) and is nowhere better illustrated than in the changing epidemiological character of poliomyelitis. As indicated above, the virus of poliomyelitis is widely disseminated in the human population, the vast majority of infections are asymptomatic or abortive, and the paraltytic disease is a relatively rare occurrence.

The epidemic described by Medin in 1891 in Sweden is regarded as one of the first epidemics to occur, and the first major epidemic in this country occurred in 1916. Beginning about the turn of the century the disease began to shift from an endemic form. characterized by sporadic cases, to a form in which cases occur in epidemics. The endemic form continues to persist in primitive populations, particularly in tropical areas,2,129 but epidemics have begun to occur in parts of Africa and India, and Japan appears to be in transition from the endemic to the epidemic form of the disease. Coincident with this shift in the character of the disease, the peak in age incidence has shifted from the 0to 4-year age group to the 5- to 9-year age group, and increasing numbers of cases occur in higher age groups and in adults.

This change in epidemiological char-

acter appears to be a consequence of the operation of social and economic factors which postpone the time of primary exposure to the virus. 65, 121 Under relatively primitive conditions, transfer of infection occurs more readily than under more enlightened hygienic circumstances. Primary infection may well occur early enough that passive immunity of maternal origin modifies, in the aggregate, the early immunizing infections, with the result that a pseudoracial immunity develops, and the population is immune to epidemic disease without experiencing clinical disease in epidemic form. By postponement of the first infection, however, the threshold density of susceptibles is raised to a point at which the population becomes susceptible to epidemic disease.

It is sometimes possible to interfere successfully with the development of population susceptibility to epidemic disease by prophylactic immunization. In diphtheria, for example, while the proportion of susceptibles constituting the threshold density is not known with precision, prophylactic immunization of 70 per cent of school age children suffices to prevent epidemics of that disease. Similarly, in poliomyelitis, the development of effective immunizing agents and their application on a sufficiently wide scale may also result in the reversion of this



*PARALYTIC CASES PRIOR TO 1951 ASSUMED TO BE 50% OF TOTAL.
SINCE 1951, CASES REPORTED AS UNSPECIFIED WERE PRORATED
AMONG PARALYTIC AND NONPARALYTIC CASES.

Figure 282. The annual incidence rates of poliomyelitis in the United States as indicated by cases reported per 100,000 during the period 1935-1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare.)

919 **IMMUNITY**

disease to the endemic form characterized by sporadic cases. A trend in this direction, following the introduction of immunization on a wide scale in 1954, is suggested by a rise in the relative incidence of the disease in the 0- to 4-year age group in 1956. Other factors may be operative also; there is, for example, evidence⁴⁸ of a similar trend in some areas in 1948 prior to immunization which may be due to factors such as changing birth rates and the incidence of disease in prior years.

The seasonal incidence of the disease, characterized by a peak in later summer and early fall, was for a time thought to suggest an insect vector. Although virus may persist in muscoid flies under experimental conditions and virus has been isolated from flies having access to human feces during an epidemic, fly control programs have not affected the incidence of the disease, and other epidemiological evidence does not indicate that the infection is transmitted by an insect

vector.

Experimental infections. For many years it was believed that only primates could be experimentally infected with poliovirus. Of such animals available for experimental purposes, the chimpanzee is the most susceptible and has, in fact, been infected accidentally in the laboratory, and the disease in this animal resembles the human disease closely. Of the commonly used monkeys, the cynomolgous monkey may be infected by the gastrointestinal route, and the rhesus is more resistant but may be infected by intracerebral inoculation. The findings at autopsy are similar to those observed in man, with pathological changes, including neuron destruction and perivascular infiltration in the gray matter of the cord.

In 1939 the Lansing strain of virus was adapted from the monkey to the cotton rat and then to mice, and later strains of the other two immunological types have been adapted to mice by introduction of the virus directly into the spinal cord. In such rodents the incubation period is two to 10 days, there is paralysis of one or more legs, few recover, and death occurs from respiratory paralysis. Type 2 strains will also infect hamsters, and one such strain, after many passages in suckling hamsters, has been adapted to the chick embryo. 152

Immunity. The immune response to poliovirus is demonstrable as the presence of complement-fixing and neutralizing antibodies in the serum and a resistance to second inoculation. Serum antibody fixes complement in the presence of tissue culture antigen, but its specificity may overlap the serotypes of the virus, possibly in part because of an anamnestic heterotypic antibody response. Complement-fixing antibody declines relatively rapidly, disappearing in from within a few months to two years. Neutralizing antibody may be titrated in the monkey, but much more readily in tissue culture. Neutralization of the virus is indicated by the lack of CPE or by changes in pH shown by the inclusion of phenol red in the tissue culture fluid. In contrast to complement-fixing antibody, it persists in detectable titer for years. Both kinds of antibody appear quite early in the disease, but if acute phase serum is taken early enough, a rise in antibody titer may be shown to occur between this serum and a convalescent serum taken three to four weeks after onset of the disease.

The presence of neutralizing serum antibody is associated with an effective immunity to the disease. As indicated above, the proportion of individuals showing neutralizing antibody increases sharply with age, and the great majority of adult serums are found to contain antibody. At one time this was thought to represent a "maturation" immunity but, as in many other infectious diseases of childhood, it is now clear that the immunity is a specific response to repeated symptomless or abortive infections which need not progress beyond the alimentary, or possibly viremic, stages to induce the immune response.

Although second, and even third, attacks of poliomyelitis have been found to occur in the same individual, it is probable, although difficult to prove in man, that the second attack is caused by an immunological type of virus different from that of the prior infection. Monkeys recovered from one attack of the disease are solidly immune to inoculation with the same type of virus, but not to heterologous types, and it is possible that this is true in man also. Naturally acquired active immunity in man is probably an immunity to all three types of poliovirus, continuously reinforced by repeated exposure and possibly symptomless infection.

Prophylaxis. Artificially acquired prophylactic immunity may be either passive or active in nature. The source of antibody in the first instance is the concentrated antibody present in pooled γ -globulin. Passive immunization has been shown to be effective in experimental animals and in man has been found to reduce the number of cases of paralytic disease by more than half, providing that the globulin is administered within the first few days of the incubation period, ⁷⁰ but serum therapy of the established disease is ineffective. Passive immunity disappears rapidly, and is no longer effective after about three weeks.

Active immunization. Active immunity may be produced by parenteral inoculation with inactivated vaccine prepared from monkey kidney cell culture, or by infection with attenuated strains of the virus by the oral route. Both kinds of vaccines must include all three immunological types of the virus to provide broad protection.

Inactivated vaccine. Vaccines inactivated with formaldehyde were prepared by Salk¹⁷¹ and are known as Salk vaccine. This kind of vaccine was subjected to extensive field tests, beginning in 1954, and was shown to provide an effective immunity as indicated by a reduction of 70 to 80 per cent in the incidence of the paralytic disease when given as a course of three inoculations, 61, 144 and there is evidence that the disease is less severe in inoculated persons,142 but the immunity is not completely effective.14 A purified vaccine has been prepared³⁵ but its relative efficacy has not been determined. The preparation of an inactivated, yet immunologically potent, vaccine is technically complex, 116 owing to the effect of formaldehyde or antigenicity. The immunity so produced is assayed as protective serum antibody and is presumably dependent upon the presence of such antibody, i.e., such immunization does not prevent the alimentary infection¹⁰⁷ but is effective in the viremic stage and on hematogenous spread of the infection.

Living attenuated vaccine. Virus strains attenuated with respect to neurovirulence have been developed by various workers, largely Sabin, Koprowski, and the Cox-Lederle group, for use as immunizing agents. The stability of such attenuated strains with respect to virulence and associated characteristics has been subjected to intensive study¹⁸⁵ and the probability of reversion to neurovirulence appears to be very small. Vaccines prepared from such strains may be mono- or divalent and given separately to avoid overgrowth and/or interference ef-

fects, or in trivalent form, but only a single dose of a given preparation is required.

These vaccines have been tested in local areas in this country, ^{33, 170} where evaluation of large scale testing is complicated by prior Salk vaccine immunization, and in England, ¹⁴⁵ and field trial has been carried out on a large scale elsewhere, especially in the Soviet Union. ^{37, 178} The results of such testing have been excellent. ^{42, 130, 131}

In addition to the accentuated antigenic stimulus provided by proliferation of the vaccine strains in vivo to give an alimentary infection and the consequent requirement for a single oral, rather than multiple parenteral, inoculation, such immunization differs from that produced by inactivated virus in two significant respects. First, immunization is against the alimentary infection and the immunity may be tested by challenge inoculation with attenuated virus. This kind of immunity tends to depress, if not effectively prevent, dissemination of virulent virus. Second, infection with attenuated virus tends to spread in the community to immunize or reinforce the immunity of others, and there is evidence suggesting that attenuated viruses may displace virulent virus in the intestinal tract. Such a combination of effects is perhaps the basis of the efficacy of oral attenuated vaccine in arresting poliomyelitis in West Berlin at the beginning of an epidemic prevalence. 102 The vaccine also produces an effective immunity in the very young; 75 per cent in newborn and 95 per cent in infants three to six months of age has been described. 79, 127 Considerations such as these have led Sabin¹⁶⁸ to suggest that poliomyelitis may be eradicated in effectively immunized areas.

ENCEPHALOMYELITIS OF MICE

A spontaneous encephalomyelitis of mice of viral etiology was described, by Theiler in this country, by Gildemeister and Ahlfeld in Germany, and by Iguchi in Japan, in the 1930's, which is characterized by a flaccid, poliomyelitis-like paralysis. The infection is widespread in laboratory mice. The virus is present in the intestinal tract and mesenteric lymph nodes of most mice four to six weeks of age and is shed in the feces, but can no longer be found at about six months, and the mice are immune. The dis-

ease can be transmitted to uninfected mice by intracerebral, intranasal, or intraperitoneal inoculation. Following intracerebral inoculation, a flaccid paralysis develops after an incubation period of five to 30 days, and the histopathology includes perivascular infiltration in the central nervous system and necrosis of the ganglion cells of the anterior horn followed by neuronophagia. 110 Rabbits and monkeys are not susceptible, and symptomless infection is produced in suckling hamsters following intracerebral inoculation.

The causative virus is immunologically distinct from the human polioviruses, but it resembles them, not only in the disease produced, but also in its small size, 25 to 30 m μ , and in its resistance to ether and glycerol. The original virus found by Theiler is called Theiler's virus, or TO virus. Two other strains found later, FA and GD7 strains, appear to be less widely distributed. So far as is known, these viruses are nonpathogenic for man. They do constitute an artifact in the use of mice for virus studies; for example, the rodent-adapted strain of Lansing poliovirus became contaminated with FA virus in the mouse and was purified

by passage in the monkey, which is not susceptible to FA virus infection.

TESCHEN VIRUS (Porcine Encephalomyelitis) 34

A highly fatal disease of swine, characterized by a flaccid, ascending paralysis, was first described in the Teschen district of Czechoslovakia as Teschen disease, and spread over Europe under various names such as Talfan disease in England and poliomyelitis suum in Denmark. It is not known to occur in this country. The causative virus is smaller than the polioviruses. 10 to 15 mu in diameter, and is immunologically unrelated to the polioviruses and also to many other neurotropic viruses infecting man. Apparently only the pig is susceptible to infection, and the disease is a diffuse encephalomyelitis affecting the cerebral cortex, the cerebellum, thalamus, basal ganglia, and anterior horn cells of the spinal cord in sharp distinction to the selective localization of the lesions produced by poliovirus.

The Coxsackie Viruses 99, 119

The first of what proved to be a new group of viruses was isolated by Dalldorf and Sickles in 1948 from two children, ill with disease diagnosed as poliomyelitis, by the inoculation of infant mice. Ten additional strains were isolated by Dalldorf the following year, and it was evident that some of the isolates differed from one another. Similar viruses were subsequently isolated by many investigators in various parts of the world. These viruses make up the somewhat heterogeneous group known as the Coxsackie viruses.* or C viruses.

These viruses have been found most often in feces and in sewage and are isolated from time to time from the throat.⁶⁴ They have been found together with poliovirus and during poliomyelitis epidemics in persons not infected with poliovirus. They appear to be most prevalent in the late summer and early

fall; human infections seem to be quite common, as indicated by the presence of antibody to all of the immunological types of these viruses in pooled antibody such as γ -globulin; and they are found in man both in association with disease and in healthy persons

Although of similar size, 15 to 35 m μ , the Coxsackie viruses are distinct from the polioviruses in pathogenicity and immunological character. They are relatively stable over a wide range of temperature and pH; preparations at near-neutrality are still active after long periods at room temperature, and for at least one day at pH extremes of 2.3 and 9.4. They are relatively resistant to substances such as ethyl alcohol, ether, glycerol, and phenolic compounds, but 0.3 per cent formaldehyde is an effective disinfectant.

Infection of cynomolgous monkeys and chimpanzees by the oral route results in a transient febrile reaction, virus is found

^{*}The first strains were found in Coxsackie, New York.

in the pharyngeal secretions and stools, there is a specific immune response, and some strains may produce neuron damage on intracerebral inoculation. Of the more common experimental animals, only the newborn mouse and hamster are susceptible to infection, although some strains occasionally infect adult mice to produce pancreatitis. While this limited host range is a definitive characteristic of the Coxsackie viruses, it does not differentiate them from certain other viruses, the Sinbis and dengue viruses (Chap. Forty), and certain tissue culture-passed strains of ECHO viruses (see below).

Virus types. These viruses are separable into two groups, designated A and B, on the basis of their pathogenicity for the infant mouse and ability to grow in tissue culture, and into immunological types within these groups. The characteristic effect of the viruses of group A in the infant mouse is an inflammatory and degenerative lesion of striated muscle without adverse effect on other tissues. Infected mice show a generalized weakness, a flaccid paralysis develops, and death occurs three to four days after inoculation. At autopsy the skeletal muscles appear white and firm, and a hyaline degeneration is found on microscopic examination.

In contrast, infection with group B viruses

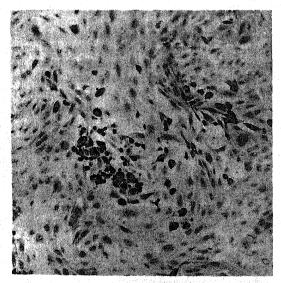


Figure 283. Coxsackie virus type B2 in monkey kidney epithelial cell culture. There are focal areas of cytopathology produced 24 hours after inoculation of the virus; the affected cells are rounded and contain crescentia and pyknotic nuclei, and the cytoplasm shows intense eosinophilic staining. (Enders: Ann. Intern. Med.)

characteristically results in lesions of the central nervous system, with focal leptomeningitis and perivascular lymphocytic infiltration, necrosis of the acinar tissue of the pancreas, hepatitis, and a necrotizing steatitis of the interscapular, cervical, and cephalic pads, and some strains produce focal lesions in the muscles, especially after intraperitoneal inoculation. The occurrence or predominance of these kinds of lesions is variable from one strain of virus to another and is also affected by the age of the mice. Irregular tonic contractions of the muscles prior to death may result in symptoms of "rolling disease" similar to that produced by some of the pleuropneumonia-like organisms (Chap. Twenty-seven). Passage in the infant mouse may be required before the pathological picture becomes characteristic, and the distinction between the A and B groups is not necessarily sharp because of variations in the pathogenicity of virus strains.

There is also a difference between the two groups of Coxsackie viruses with respect to growth in tissue culture. Those of group A are, in general, not cultivable in tissue culture, but the A9 type is an exception, and will grow in monkey kidney tissue culture and in some other kinds of tissue culture also, and has frequently been isolated by this method. Some strains of type A9, isolated in tissue culture, have also been found to be nonpathogenic for infant mice, thus resembling the ECHO viruses. Some group B viruses are cultivable in various kinds of tissue culture, but there is some irregularity in the pattern of susceptibility. Monkey kidney tissue culture, prepared in monolayers, appears to be one of the most generally satisfactory for viruses of group B, and cytopathic effects are produced.

These viruses are also immunologically heterogeneous and, although there may be some overlapping, there appears to be no common antigenicity to bind these viruses together as a group. They are, rather, immunologically individual. A total of 23 specific serotypes has been defined within group A, and six serotypes within group B, and no doubt additional serotypes will continue to be described. These serotypes are given arbitrarily assigned numbers, to give type designations of A1, A2, etc., and B1, B2, etc.

These types are separable by comple-

ment fixation, and by neutralization and protection tests, with substantially the same results. Infected muscle tissue is used for the preparation of immunizing and complement-fixing antigens, and a partial purification of tissue debris may be effected by centrifugation. Protective antibody is demonstrable by the neutralization technique, but infant mice cannot be actively immunized and subsequently challenged because the natural resistance to infection appears very early. An effective passive immunity occurs in infant mice born of and nursed by actively immunized mothers. The protection conferred by active immunity is demonstrable in the chimpanzee as an immunity to challenge inoculation.

The disease in man. As indicated above, serological evidence indicates a wide prevalence of human infection with Coxsackie viruses, and these viruses have been associated with human illness and found in healthy persons. Their association with poliomyelitis appears to be purely fortuitous, and various attempts to demonstrate a potentiating effect of Coxsackie virus infection on poliomyelitis have given negative results. In fact, an interference between some group B strains and poliovirus occurs in tissue culture, and it has been suggested that Coxsackie virus infection may, on occasion, tend to exclude poliovirus infection.

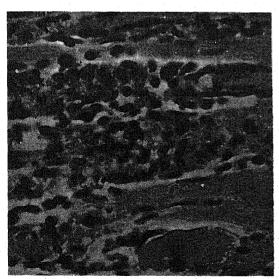


Figure 284. Lesion in striated muscle of a mouse infected with Coxsackie virus. Two sarcolemmic tubes with numerous mononuclear phagocytes and remnants of hyaline material within the sarcolemmic sheaths. Demarcated segmental involvement is apparent. × 500. (Godman, Bunting, and Melnick: Amer. J. Pathol.)

There are, however, some clinical entities with which Coxsackie viruses have been found to be in close, and possibly etiological, relation. These include febrile pharyngitis and herpangina, epidemic pleurodynia, some portion of cases of aseptic meningitis, and myocarditis. The Coe virus, originally isolated from mild upper respiratory disease, has been found to be Coxsackie A21 virus. 176

Herpangina. This is a febrile disease, usually seen in children, which occurs in both epidemic and sporadic form in many parts of the world. There is often vomiting and abdominal pain, and the characteristic lesion is a small, herpes-like vesicle which breaks down to leave a shallow ulcer. These lesions occur on the edge of the soft palate. on the tonsils, in the pharynx, and on the anterior pillars of the fauces. Recovery is usually prompt, within two to three days. and complete. Coxsackie viruses have been isolated from as many as 84 per cent of cases of herpangina examined, and six serotypes, A2, A4, A5, A6, A8, and A10, have been found for the most part, although not exclusively.

There is circumstantial and other evidence, including laboratory infection, which indicates that the group A viruses can produce a febrile pharyngitis, particularly in summer, without the identifying herpangina lesion, giving the disease sometimes known as three-day fever or summer grippe. When the infection takes this form and occurs during epidemics of poliomyelitis, it may be diagnosed as abortive poliomyelitis. Russian workers have, for example, reported a new type of poliovirus, type 4, which was subsequently shown to be Coxsackie virus type A7.81, 194 Or, more severe cases of disease have been observed in which the herpangina is associated with parotitis.82

Epidemic pleurodynia. 10, 56, 165 This disease was first observed in Bornholm, Denmark, in 1930, and is also known as Bornholm disease, epidemic myalgia, and "devil's grip." The disease tends to occur in epidemic form, most commonly in late summer. The incubation period is two to four days, the onset sudden, and the disease is characterized by acute pain in the thoracic wall, abdomen, and lower region of the back and is commonly accompanied by headache, but there are no respiratory or constitutional symptoms. The pain is paroxysmal, lasting a day or more, and the muscles are tender to

pressure. There is a tendency to relapse at intervals of a few days or longer, and orchitis and meningeal involvement may occur.

Strains of group B Coxsackie virus have been isolated from the throat or feces in a considerable portion of cases in several epidemics studied, increase in antibody titer to the virus isolated is observed, and it seems probable that these viruses may be the etiological agents of this disease.

Aseptic meningitis. Aseptic meningitis is a clinical syndrome rather than an etiological entity and may occur in epidemic form. 164, 186 It is usually mild, with signs and symptoms indicating involvement of the meninges. Fever, headache, nausea, pain in the abdomen, and stiffness of the neck or back usually occur. Pleocytosis may be present, up to 100 cells per cu. mm. being observed in the spinal fluid. This disease may be caused by a variety of nonbacterial agents, including Coxsackie viruses. Viruses of

group B—including serotypes B1, B2, B3, and B4, and one of group A, A9, which is set apart from the other group A viruses by its cultivability in monkey kidney tissue as noted above—have been isolated from the feces and from the spinal fluid in cases of this disease. Neutralizing antibody to the isolated virus occurs in the serum, and it is probable that some portion of cases of aseptic meningitis are Coxsackie virus infections.

Myocarditis. 108 In recent years cases of acute myocarditis of infants have been described from various parts of the world. In a few instances isolations of group B Coxsackie viruses have been reported, including recoveries from affected myocardium and from spinal cord. In the latter case, an infant that became ill on the third day after cesarean section and died on the seventh day, serotype B3 virus was found, and the circumstances suggested that intrauterine infection may have occurred.

The Enteric Orphan Viruses 40, 87, 99

With the development of practical and generally applicable methods for the isolation of polioviruses from the intestinal tract in tissue culture, a great many isolations of cytopathogenic agents other than poliovirus have been made. These viruses are previously undescribed entities and, while some have been found in cases of poliomyelitis, often diagnosed as the nonparalytic disease. in many instances there has been no specific association with illness, and they have been called orphan viruses. Those isolated from man have been named the ECHO, or enteric cytopathogenic human orphan, viruses; those from monkeys the ECMO viruses: those from cattle the ECBO viruses; and those from swine the ECSO viruses. Similar viruses isolated from dogs may make up another group of ECDO viruses. Of these, the ECHO viruses have been of the greatest interest, and, with their characterization, some at least have been found to occur in association with, and possibly etiological relation to, human disease.

ECHO VIRUSES

These viruses are similar to polioviruses and Coxsackie viruses in size and have been

found to be about 30 m μ in diameter with the exception of one, ECHO type 10, which has been reported to be 60 to 90 m μ in diameter. They are differentiated from one another by their host range and pathogenicity as expressed in tissue culture, including plaque morphology in monolayer culture, and by separation into antigenic types. Some, though not all, form hemagglutinin²⁸ and are related to one another in this respect, but not to the influenza virus and other myxoviruses.

Culture. In general, the ECHO viruses grow more readily on primary isolation in monkey kidney tissue culture than on HeLa cell culture. This is in contrast to the adenoviruses, which are usually more readily isolated in HeLa cell culture. Many strains of ECHO viruses have been grown in HeLa cell culture, with the appearance of cytopathic effects and complement-fixing antigen in the culture fluid, but the virus titer reached is not as high as that in monkey kidney tissue culture. 6, 125

Plaque morphology. Growth in kidney tissue culture from a variety of species of monkeys has been studied in detail, 84, 85, 86 as has the comparative susceptibility of the cells to these and to poliomyelitis and Cox-

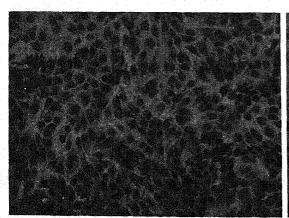
Differentiation	- 0	W3	₹70	•	TIPLE	C-14
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				PLAQUE FORMATION	
VIRUS	SEROTYPE	PLAQUE MORPHOLOGY	RHESUS CELLS PATAS CELLS		
Poliomyelitis		1, 2, 3	Large, circular, sharp boundaries	+	
Coxsackie	B A	1, 2, 3, 4, 5 9	Large, circular, diffuse boundaries, slow development		
ЕСНО	A B	1, 3, 4, 6', 9, 11, 13, 14 7, 8, 12	Small or medium size, irregular, slow development Large, circular	+ + +	

sackie viruses, assayed by the rate of development and morphology of plaques produced in monolayer cultures. It will be recalled that host cell susceptibility and plaque morphology have been established as valid differential characteristics, inheritable and genetically determined, in the case of bacterial viruses (Chap. Three and Seven), and may be regarded as analogous to the morphology of pocks produced on the chorioallantoic membrane by poxviruses and to bacterial colonial morphology on differential mediums. Of the various species of monkeys investigated, the kidney tissue of two, the rhesus and patas (Erythrocebus patus, the African red grass monkey), have been of considerable differential value.

On monolayers of rhesus cells, the polioviruses produce circular plaques with clear centers and sharp boundaries as illustrated in the accompanying figure. The plaques formed by the Coxsackie viruses which are cultivable in tissue culture, group B and A9, develop more slowly, and the boundaries are not sharp owing to the persistence of living cells at the periphery. The plaque-producing ECHO viruses are separable into two groups. One, designated group A and containing serotypes 1, 3, 4, 6', 9, 11, 13, and 14, produces slowly developing, small to medium size, irregular plaques, and group B, consisting of serotypes 7, 8, and 12, produces large circular plaques.

The susceptibility of the two kinds of monolayer kidney cell cultures is also differential. Poliovirus and ECHO group B form plaques on both kinds of cells, but the Coxsackie viruses and ECHO group A form plaques on rhesus cell cultures but not on patas cell cultures. There is further differential susceptibility within the ECHO viruses in that serotypes 2, 5, and 6 do



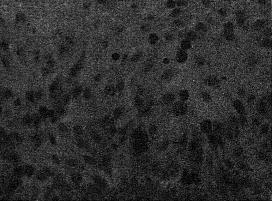


Figure 285. The cytopathic effect of ECHO virus type 1 in culture of monkey kidney epithelial cells. Left, uninoculated six-day culture of cells; right, 24 hours after the inoculation of virus, a few rounded cells are scattered throughout the field. Carnoy fixation; hematoxylin and eosin. (Melnick: Adv. Virol.)

not produce plaques on either kind of cells, and they are cytopathogenic for rhesus cells but not for patas cells in tube cultures with fluid overlay.

Antigenic types. The ECHO viruses are separable into serotypes by cross-neutralization tests in tissue culture, and standard antiserums are used for identification.⁹⁷ The test is made sharply specific by titration of the 50 per cent tissue culture dose, the TCD₅₀, and the prototype strains have been numbered on an arbitrary basis.

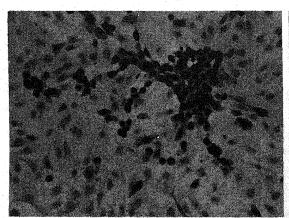
New serotypes continue to be described, to give a total of 33 by 1965. These may be described under other names and subsequently assigned to the ECHO group. For example, the JV viruses¹⁵⁹ are considered to be ECHO viruses, the JV-4 strain being ECHO-25; the Puerto Rican strain PR-10 is ECHO-32;²⁴ and that originally described as Toluca-3 is ECHO-33.¹⁵⁸ Similarly, the respiratory viruses, JH and 2060, were subsequently designated ECHO-28, and still later assigned to the newly described rhinovirus group (see below) and considered to represent type 1 of this group.

Strains within a serotype are not necessarily antigenically identical, and those with broader antigenicity than the prototype strain have been called prime strains. Strains within type 6 have been studied in this connection, and the strains designated 6' and 6'' have been found to have a broader antigenic specificity than that of prototype strain 6. There are cross-reactions among types 1, 8, and 12, ECHO 4 antiserum

neutralizes adenovirus 8, and Coxsackie B4 is neutralized by antiserums to several ECHO types.

Pathogenicity.¹⁷² In a general way, the ECHO viruses are set apart from the Coxsackie viruses in their lack of pathogenicity for the infant mouse although, as noted above, occasional Coxsackie strains are not pathogenic for the infant mouse on primary isolation. While originally found in the absence, or apparent absence of specific disease, certain of the ECHO viruses have been found in association with human disease, especially aseptic meningitis.

Types 4, 5, 6, 9, and 16 have been found repeatedly in cases of aseptic meningitis in the absence of other causative agents, and types, 5, 6, and 9 have been isolated from the spinal fluid. 49, 114, 143 Epidemic aseptic meningitis occurred in Europe in 1955 and 1956, and spread to this country in the following year, in which type 9 appeared to be the etiological agent. The virus strains found were apparently antigenic variants of the prototype strain. Infections associated with type 9, and also with types 4 and 16, have often been characterized also by the presence of a rash. In view of its association with aseptic meningitis, the status of ECHO type 9 is of interest; certain of the apparently more virulent strains, i.e., isolated from spinal fluid, have been found to become pathogenic for infant mice after tissue culture passage, producing myositis and paralysis, and might be considered to be a type B Coxsackie virus.



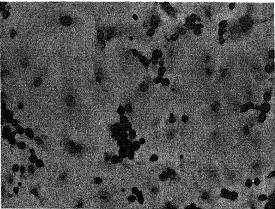
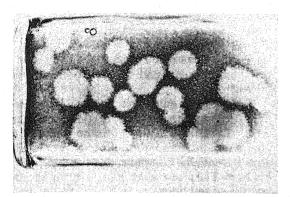


Figure 286. The cytopathic effect of ECHO virus type 1 in culture of monkey kidney epithelial cells. Left, 66 hours after the inoculation of virus; right, 90 hours after the inoculation of virus. At 66 hours there are foci of rounded, densely stained cells and infected cells scattered over the cell sheet. At 90 hours many infected cells have become detached from the glass, most of the remaining cells are rounded because of viral infection, but some normal-appearing cells are still present. Carnoy fixation; hematoxylin and eosin. (Melnick: Adv. Virol.)



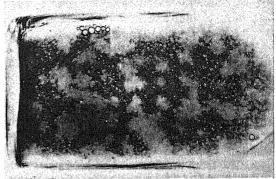


Figure 287. The types of plaques produced by poliovirus type 3 (left) and ECHO virus type 6 (right) on monolayer cultures of monkey kidney tissue. (Hsuing and Melnick: Virology.)

REOVIRUSES167, 181

As noted above, the virus of ECHO type 10 is set apart from the other viruses of this group in that it is considerably larger. It forms hemagglutinin for human O cells but not for chicken, guinea pig, sheep, or bovine erythrocytes; the red cell receptors are not affected by RDE but are destroyed by treatment with periodate. There is a distinctive cytopathic effect in monkey kidney cell culture, with granular degeneration of the cells leaving intact nuclei, and intracytoplasmic inclusion bodies are present. These and other distinctive characteristics83 have led to the separation of ECHO 10 strains as a separate group designated reoviruses, the term referring to both respiratory and enteric origin.

Strains of this group contain a common. or group-specific, complement-fixing antigen, and are separable into serotypes by neutralization and hemagglutination-inhibition tests.154 Three serotypes are distinguished. Type 1 consists of the Lang strain, found in healthy children, as the prototype and includes the simian virus SV-12, and other strains isolated from children with upper respiratory disease. Type 2 includes the D5 strain, found in a child with diarrhea, as the prototype, together with simian strains and others of human origin. Two strains have been suggested as the prototype of type 3, one the Dearing strain from a child with diarrhea and the other the Abney strain recovered from a child with febrile upper respiratory disease,160 and subsequently from nursery children. Type 3 strains have also been recovered from cattle. Types 2 and 3 have been found in wild and laboratory mice respectively. Suckling mice may be infected experimentally, some strains producing clinical disease.

ENTERIC ORPHAN VIRUSES OF LOWER ANIMALS⁹⁶

It was noted above that viruses analogous to the ECHO viruses have been found in lower animals, including monkeys, cattle, and swine. The simian viruses have been of most interest, since they are found both in the bowel³⁶ and in apparently healthy monkey kidney tissue88, 89 used for culture of the poliomyelitis and other viruses. A considerable number of simian strains have been examined for plaque morphology on rhesus and patas monkey kidney tissue culture and for antigenic specificity.84 On the first basis they are separable into three groups: group A, characterized by the delayed development of small plaques on both rhesus and patas cultures; group B, made up of strains forming large circular plaques on both rhesus and patas cultures in which islets of unaffected cells are found; and group C strains, which produce plaques on rhesus cultures similar to those of group B but do not produce plaques on patas cultures. Differentiation on the basis of plaque morphology is not necessarily consistent with antigenic and cross-neutralization is differences. observed between plaque types. So far as is known, these viruses, the ECMO group, are not pathogenic for monkeys.

The Encephalomyocarditis Viruses

encephalomyocarditis, or EMC, viruses are represented by a number of strains which appear to be substantially identical. The first to be isolated, the Columbia SK virus, was found in 1940 in a monkey which had been inoculated with the Yale SK strain of poliovirus, but it is clearly distinct, both in antigenicity and pathogenicity, from poliovirus. The MM virus was isolated in 1943 from the brain of a hamster which had been inoculated with material from a human case of paralytic disease. These two strains were at one time considered to be "mouse poliomyelitis viruses" or murine variants of poliovirus, but it is now clear that this is not true. The strain called the EMC virus was isolated in 1945 from a captive chimpanzee in Florida which had died of acute endocarditis complicated by myelitis. The Mengo virus was isolated in 1948 from a captive monkey in Uganda and later from mosquitoes (Taeniorrhynchus fuscopennatus) and a mongoose in the same area. It has also been found as the causative agent of an epizootic disease of swine in Panama. 124

These viruses are ether-resistant RNA viruses, about 25 m μ in diameter, and fall into the enterovirus subgroup of the picornaviruses. They form hemagglutinin⁴⁶ and, although antigenically closely related, strain differences are demonstrable by the HI reaction. There are also interstrain differences in plaque morphology.⁵⁷

A variety of experimental animals may be infected to produce disease ranging from severe fatal disease to inapparent infection, depending upon the species and age of the animal.100 Rapidly fatal disease is produced in the mouse, hamster, and cotton rat, disease varying in severity in the guinea pig and rhesus monkey, and inapparent infection with no more than a transient febrile reaction in the rabbit and white rat. In experimental animals the virus is pantropic, occurring in the blood and widely distributed in the tissues, including the central nervous system. Degenerative and inflammatory changes are produced in skeletal and cardiac muscle, and the lesions in the central nervous system are those of polioencephalitis, with congestion, lymphocytic infiltration, and focal areas of necrosis in the granular and Purkinje cell layers. Variants have been described which differ in their myocardiotropic and neurotropic properties.⁴⁴

The virus is cultivable in the chick embryo, killing the embryo in three or four days. In mouse muscle tissue culture a characteristic cytopathic effect is produced, with essentially complete destruction in 72 hours.³² They are also cultivable in HeLa and other cell lines.

The disease in man. Human infection with EMC virus has been observed on a number of occasions, and there is also serological evidence of infection in both man and lower animals. Soon after its isolation, a laboratory worker was infected with Mengo virus to give a meningoencephalitis of short duration, followed by rapid recovery. The virus was also isolated from four children in Germany who had indefinite illness with signs of meningeal involvement. An epidemic of mild febrile disease, called three-day fever, occurred in Manila in 1945 and 1946 among American military personnel which serological evidence showed to be an infection with this virus. In these cases the febrile period lasted only two to three days; in some cases there were mild encephalitic symptoms, recovery was prompt and without sequelae, and there was no evidence of cardiac involvement.

The presence of neutralizing antibody in both man and monkeys has been observed in serological surveys in Uganda and Tanganyika, with perhaps 3 to 4 per cent of human serums positive. 11 In this country antibody has been found in human serums with some frequency. Of one series of 300 serums examined, nine were found to contain antibody; of the nine positive serums, three were from convalescents from disease diagnosed as mild aseptic meningitis and five from patients diagnosed as having nonparalytic poliomyelitis. 195 An even higher incidence was reported in another group of serums; 21 per cent of serums from poliomyelitis convalescents and 16 per cent of "normal" serums were found to contain antibody to EMC virus.94 Also in this country it has been found that 18 per cent of rat serums examined contained antibody. 195 The presence of the virus in South America has been shown by its isolation from a monkey, Aotus trivirgatus, in Colombia and by the occurrence of antibody in man in Peru⁹³ and in Panama.⁴⁵ Monkeys may be infected by feeding mouse brain virus, but the evidence in general is taken to suggest that

EMC virus occurs as an infection in rodents¹⁰¹ which is transmissible to man and monkeys by mosquitoes and possibly in other ways as well.

The Common Cold Viruses^{5, 187, 188}

The clinical syndrome characterizing the mild upper respiratory disease called the common cold merges imperceptibly into that of acute respiratory disease which, as noted elsewhere (Chap. Thirty-eight), is of diverse etiology. Mild disease resulting from infection with the parainfluenza viruses, respiratory syncytial viruses, and adenoviruses (see below) may be indistinguishable from the common cold. Nevertheless, the common cold appears to be caused more often by viruses of the picornavirus group, which form a subgroup separable from that of the enteroviruses.^{33, 68}

Rhinoviruses.¹⁹⁰ The first of these viruses to be described were those of the JH 2060 type which, as noted above, were subsequently put into the ECHO group as the ECHO-28 serotype. Closely similar, but antigenically distinct, viruses were isolated in England at the Salisbury Common Cold Research Unit and were referred to as the Salisbury agents and in the United States by Hilleman and his co-workers in Pennsylvania, who designated them coryzaviruses, at the National Institutes of Health, and at the University of Chicago.

For a time these viruses were known as the ERC (ECHO-rhino-coryza) viruses and as the muriviruses (mild upper respiratory illness). The term rhinovirus was proposed on an international basis and is now generally accepted for this group. There is also general agreement on the identification of the serotypes making up the group by numbers based on the chronology of description. To 1967, 55 serotypes have been established and assigned numbers; of these the JH-2060-ECHO-28 virus is rhinovirus 1.

These viruses are ether-stable RNA viruses with a particle size of 15 to 30 m μ , characteristics of the picornavirus group. As a subgroup, they are separable from the viruses of the enterovirus subgroup by their acid lability (pH 3 to 5 for one to three hours) and their relative thermostability at

50° C., which is enhanced by M MgCl₂.52 Some are cultivable only in human cell (embryo kidney or diploid cells) cultures. and others also in monkey kidney and other kinds of cell cultures, with CPE manifested as the appearance of ovoid refractile cells. They are separated into two types on this basis, the H type cultivable only in human tissue culture, and the M type growing in monkey and other cell cultures. In general, growth is better in roller tube than in still cultures, and occurs at 33° C. Growth of these viruses in human embryo trachea tissue, or organ, culture occurs more readily than in other kinds of tissue culture.80 Neutralizing antibody is titrated in cell culture.

Pathogenicity. Serological surveys have shown that the distribution of neutralizing antibody in human serums is world-wide, but neutralizing antibody is not found in serums of lower animals. Presumably the reservoir of infection with the strains found in man is only man, but bovine²⁰ and equine^{53, 137} rhinoviruses have been described. The latter occur as at least two serotypes and infect man in populations at risk, such as stable workers.¹³⁹

The significance of human rhinoviruses in the etiology of the common cold has been demonstrated in a number of studies based on isolation and identification of the associated virus. The spectrum of viruses differs between infants and very young children on the one hand, and adults on the other, the parainfluenza viruses, respiratory syncytial viruses, and other myxoviruses assuming greater importance in the former. 38, 47, 149 In one study⁷⁷ rhinoviruses were found in 4.8 per cent of children and 14.2 per cent of adults, and in another⁷¹ 5 per cent and 10 per cent respectively. Similar results, 17 per cent positive isolations, have been obtained with college students,135 to give good agreement with an average figure of 15 per cent in adults. The predominance of the rhinoviruses was also shown in another study of

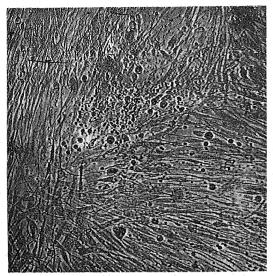


Figure 288. The cytopathic effect (CPE) produced by rhinovirus type 2 in a culture of the W38 strain of human diploid cells. Unstained. (Hamre.)

college students over a period of four years, in which 80 per cent of the total isolations were rhinoviruses of 50 serological types, and of the remainder, most (15 per cent) were myxoviruses.⁷²

Failure to obtain a higher proportion of positive isolations from cases of clinically apparent disease has been attributed to the relative insensitivity of the culture methods, or the occurrence of other as yet unknown viruses not recoverable by the methods used. Support for the former is provided by the higher proportions, nearly twice as many, of positive isolations when embryonic human trachea tissue culture is used. 189

It is probable that the virulence of the rhinoviruses is not great and that most adults have some degree of partial immunity as indicated by serological surveys. Studies on the occurrence of, and immune response to, rhinoviruses 1 and 2 in a military population over a 24-week period (January to June)⁹² showed that both viruses were present during five of the weeks, and one or the other during 16, with isolation rates of 12 per cent. The incidence of positive isolations in those with significant antibody titer was only 20 per cent of that in those persons showing no antibody. It is probable that the respiratory viruses, including the rhinoviruses, persist at a low level of infection, with the development of clinical disease attributable in very considerable part to nonspecific stress.⁴

Immunity. As indicated above, there is an association between pre-existing antibody and resistance to development of disease. Immunization with virus vaccine by the intramuscular route followed by intranasal challenge of small numbers of persons showed an approximate 10-fold reduction in colds in the vaccinated group over those occurring in the control group. 148 Other studies on the effect of naturally occurring vaccine- or infection-induced body response have also shown an approximate correlation between serum antibody levels and occurrence and/or severity of disease produced by challenge inoculation,30 though some irregularities were observed. It has also been generally observed that virus is isolated less often from persons showing serum antibody and such persons tend to carry and disseminate the virus less frequently. While it seems clear that an appreciable degree of immunity to a given virus serotype occurs and can be produced by immunization, in view of the multiplicity of serotypes of rhinoviruses as many as 40 were found in one study⁷¹ and 50 in another⁷² – the practical efficacy of prophylactic immunization to the common cold as a disease entity remains somewhat uncertain.

Foot-and-Mouth Disease²⁷

Foot-and-mouth disease is a naturally occurring, highly contagious disease of cloven-footed animals, primarily of cattle, swine, sheep, and goats, which occasionally infects man. It is characterized by a vesicular eruption on the lips, buccal cavity, pharynx, and extremities, but the causative

virus is quite unrelated to the herpes viruses.

The virus is present in the vesicle fluid early in the disease and may also be present in the blood, saliva, urine, and milk, but disappears within three to five days from the local lesions and the blood. This was the first animal disease shown to be of virus etiology,

by Löffler and Frosch in 1897. The virus is an ether-resistant RNA virus, 22 to 23 mu in diameter,8 and is considered to be a member of the picornavirus group. Like the rhinoviruses, it differs from the enterovirus subgroup in its relative instability at acid reactions. It has been grown in swine and lamb kidney, bovine kidney, and other tissue cultures, and may be assayed by the plaque method.9 Neutralizing and complement-fixing antibody are formed by the infected animal, the former assayed by inoculation into the bovine tongue, and the antigen in the latter is infected epithelium of the tongue. The virus occurs as seven main serological types, three of which have a number of subtypes.

The disease may be reproduced in the guinea pig, usually by intradermal inoculation of the footpad. A vesicular eruption occurs at the site of inoculation, constitutional symptoms appear, and a secondary eruption appears on the tongue, gums, and lips. The eruption begins to subside about

two days after its appearance, and the lesions are healed in about two weeks. Infected animals are debilitated, but the disease is relatively mild in that the case fatality rate is 5 per cent or less.

The disease in cattle and other domestic animals is of considerable economic importance, not because of its fatality rate of 2 to 3 per cent, but because of its debilitating effect, with consequent loss in milk and meat production. Immunization has not been a satisfactory or practical control measure, and the disease is controlled by slaughter of infected animals and rigorous quarantine, the latter including certain meat products such as frozen beef in which the virus remains infective. The virus persists in dry form and may be transported mechanically on clothing, by wild birds, etc. Human infection appears to be very rare, and the disease is relatively mild and of minor significance in man except in relation to its spread among domestic animals. 136

Viral Diarrheas

Diarrheal disease of viral etiology occurs in both man and lower animals. ECHO virus type 18 has been found to be associated with summer diarrhea of infants.⁵⁹ Other epidemic diarrhea of the newborn may also be of viral etiology, and there is some evidence suggesting that the disease may be transmitted to calves, but the causative agent is poorly known.

Viral diarrhea, or epidemic nonbacterial gastroenteritis, occurs in adults also and has been studied at length by Gordon and his colleagues.67 It has been found that at least two kinds of disease occur, a febrile and an afebrile type. The afebrile type has an incubation period of two to three days and is characterized by nausea, vomiting, abdominal spasms, and watery stools, and an attack results in an immunity which persists for a year or more. It is associated with a filtrable agent, Marcy, which has been transferred through eight serial passages in human volunteers, but disease has not been produced in experimental animals. The agent associated with the febrile disease, which has a shorter incubation period and a less protracted course, has been designated FS, and there is evidence that this and the Marcy agent are immunologically distinct. The Marcy agent appears to be closely related to, if not identical with, the agent responsible for nonbacterial infectious diarrhea in Japan.⁶²

Diarrheal disease of viral etiology is not uncommon in lower animals. One of the most familiar of these to the laboratory worker is the epidemic diarrhea of suckling mice which occurs in explosive form, more commonly in the winter months. Intranuclear inclusion bodies are found in the epithelial cells of the villi, but the nature of the etiological agent is not well established. Calf pneumonia enteritis is of established viral etiology, the virus has been isolated, and the disease transmitted to susceptible newborn animals.25 The psittacosis agent may cause diarrheal disease in older animals, and three other immunologically distinct agents, presumably viral in nature, may also cause the disease. Transmissible gastroenteritis of swine is also of viral etiology, and the virus has been isolated and characterized immunologically. 69

Viral Hepatitis^{58, 104, 147}

Liver damage with icterus occurs in infectious diseases such as leptospirosis and yellow fever, but there is, in addition, hepatitis of specific viral etiology. There are two kinds of this disease, the one infectious hepatitis and the other serum hepatitis, which are caused by different viruses and differ in epidemiological character.

INFECTIOUS HEPATITIS

Infectious hepatitis (infective hepatitis, epidemic jaundice) is a subacute disease of world-wide distribution which tends to occur in children and young adults. It may appear in epidemic form within households and institutions and in military personnel, as in the two World Wars and the war in Korea. The prevalence of the infection is not precisely known, and it is probable that many cases are subclinical.

The incubation period varies from one to six weeks, with an average of perhaps three to four weeks. The disease is one of diffuse involvement of the liver and is separable into two stages, the pre-icteric and the icteric. The onset may be abrupt or insidious, and the symptoms include fever, gastrointestinal distress, headache, anorexia, and lassitude; the postcervical lymph nodes are often involved, and there is

leucopenia with relative lymphocytosis. This pre-icteric phase may persist for as long as three weeks but usually lasts for only a few days. Symptoms tend to subside, but there is exacerbation of some, especially those of abdominal discomfort, with the appearance of jaundice, and the liver and spleen are palpable and tender. Examination of biopsy specimens indicates progressive liver damage during the course of the disease, with relatively extensive parenchymal destruction by the time jaundice is apparent, but subsequently regeneration occurs which is complete in most cases by three months. Consistent with this, there is early evidence of hepatic dysfunction, and bilirubinuria occurs toward the end of the pre-icteric phase of the disease.

The icteric phase may persist for some weeks and is usually followed by uneventful convalescence. The case fatality rate is lower in children than in adults and has been reported at less than 2 per 1000 in military personnel. Relapse occurs with variable frequency, however, ranging from less than 1 per cent to as much as 18 per cent of cases. In many instances relapse is evident only as some degree of hepatic dysfunction, but in others the icteric phase of the disease recurs, although often in less severe form. In some few cases impairment of hepatic function may persist over protracted periods,

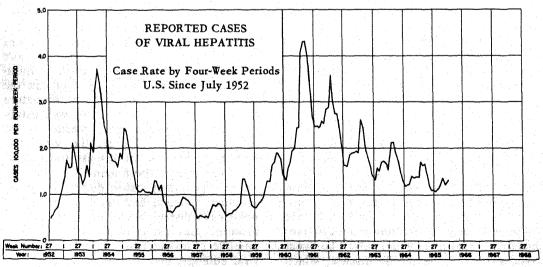


Figure 289. Cases of viral hepatitis, both serum and infectious, in the United States during the period 1952-1965. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare.)

and in some small portion of these, symptoms may persist also. Rare complications of the disease include pneumonia, meningitis, and myelitis. A startling and provocative association between the prevalence of hepatitis and that of mongolism nine months later, with a periodicity of five to seven years, has been described in Australia; it has been suggested that subclinical infection of the aging mother at or on about the time of conception might affect the chromosomes of the ovum.¹⁸²

Hepatitis virus A. It is customary to designate the virus of infectious hepatitis as hepatitis virus A to distinguish it from the virus of serum hepatitis. It has not been possible as yet to infect experimental animals although there is evidence suggesting that chimpanzees may have been a source of human infection. 78, 146 There are a good many reports of isolation of a viral agent in embryonated eggs or one or another kind of tissue cell culture, 39, 50, 106, 109, 151 none of which has as yet been accepted as an etiological agent of the disease. 112, 115 Information is limited to that obtained from the inoculation of human volunteers.

The virus passes bacteria-proof filters such as the Seitz EK and is relatively resistant, for it is not inactivated at 56° C. for 30 minutes or by concentrations as high as 1 ppm of residual chlorine in the same time. It is present in the blood and feces in the pre-icteric or early icteric stages of the disease and has been found in the feces during the incubation period, as much as two to three weeks before the onset of jaundice,105 but attempts to demonstrate the virus in urine and in nasopharyngeal secretions have given equivocal or negative results. Man may be infected by either parenteral inoculation or ingestion of the virus, and it is probable that the latter route is involved in most naturally-occurring infections. 122, 141 Waterborne, milkborne, and foodborne epidemics have been described, and other more direct forms of contact are indicated by epidemiological evidence. The occurrence of the disease in the winter months suggests respiratory spread of the infection, but this is not consistent with transmission of the disease by biting insects even though the blood is highly infectious.

The relatively greater prevalence and severity of the disease in the lower age groups, including young adults, suggests that the infection may be relatively prevalent and produces an effective immunity. Pooled γ -globulin has a definite protective effect when given during the incubation period. Effective immunity has also been demonstrated experimentally with human volunteers, but there is some reason to believe that all strains of the virus are not immunologically identical although cross-protection occurs between strains. Hepatitis virus A is antigenically distinct from the virus of serum hepatitis. Second attacks of hepatitis occur, but the lack of a specific diagnostic test makes it difficult to assess their significance.

SERUM HEPATITIS

The disease serum hepatitis has probably been prevalent for many years, and possibly epidemics of jaundice associated with vaccination and the chemotherapy of syphilis were of this nature. It came into prominence in the early 1940's in connection with the immunization of military personnel to yellow fever with an immunizing agent which included human serum. Jaundice appeared to be associated with some, but not all, lots of serum, and with the elimination of serum from the vaccine, cases of so-called postvaccinal jaundice ceased to occur. Subsequently, there have been many instances of icteric disease following the injection of human serum or plasma, or blood transfusions, all indicating the presence of an infectious agent in human blood.

The incubation period of serum hepatitis is relatively long, eight to 22 weeks, and the disease is indistinguishable clinically from infectious hepatitis after the onset of symptoms. It has not been possible to transmit this disease to experimental animals, and information about the virus has been derived from experimental inoculation of human volunteers.

Hepatitis virus B. The causative virus is named hepatitis virus B to distinguish it from hepatitis virus A of infectious hepatitis. The two viruses have similar properties in that both show substantially the same resistance to heating, and activity persists in blood, serum, and plasma over extended periods. They are, however, immunologically distinct, there appears to be no crossimmunity between them, and they may be found together in both place and time. Hepatitis virus B is present in the blood, not only during the disease, but also in the

incubation period, and a chronic carrier state has been observed in which the blood may be infective for many months. It is not found in nasopharyngeal secretions, and it differs from hepatitis virus A in that it is not present in the feces, and man is not infected experimentally by the gastrointestinal route.

It is quite uncertain how the infection persists under natural conditions, but it is readily transmitted to man by inadequately sterilized syringes and needles used for purposes such as the administration of chemotherapeutic drugs, convalescent serum, or insulin, the withdrawing of blood, procedures such as tattooing, and the administration of infectious blood, plasma, or certain blood products such as fibrin foam and thrombin. Hepatitis virus A may also be transmitted in this way.

As in infectious hepatitis, antibody concentrates such as γ -globulin are prophylactic, and an attack of the disease appears to confer an appreciable degree of effective immunity. The virus is rarely found in γ -globulin; such preparations are possibly contaminated, but the virus may be neutralized by the relatively high concentration of antibody.

The persistence of the virus in blood, plasma, and serum poses a considerable practical problem in view of the lack of any method, other than human inoculation, of demonstrating its presence. It is destroyed by ultraviolet irradiation under experimental conditions, but this is not a practical method for the treatment of blood or plasma. and chemical sterilization, as by nitrogen mustards, has not yet been successful. Allen and his co-workers3 have found that the incidence of hepatitis in recipients of plasma is markedly reduced when the fluid plasma is allowed to stand at room temperature for three to six months, suggesting an appreciable degree of inactivation of the virus.

HEPATITIS OF LOWER ANIMALS

Hepatitis of viral etiology occurs in lower animals, including the dog and mouse, and a similar disease has been described in guinea pigs and ducks. 95 Murine hepatitis has been of interest as a disease possibly analogous to viral hepatitis of man.

Canine hepatitis. An infectious hepatitis of dogs was described in 1947 by Rubarth in

Sweden as caused by a virus related to the fox encephalitis virus; it is sometimes known as Rubarth's disease. The disease ranges in severity from symptomless infections to an acute fulminating fatal disease, with death occurring within 24 hours. The disease may be reproduced in susceptible puppies, but not in the usual experimental animals. Intranuclear inclusion bodies are found in the liver cells and vascular endothelium, and the virus passes bacteria-proof filters. The virus has been cultivated in the yolk sac of embryonated eggs and in raccoon kidney tissue culture, reproducing the disease in susceptible puppies after 12 passages in the former and after 39 passages in the latter. A lyophilized antigen prepared from infectious liver tissue fixes complement in the presence of serum from convalescent dogs, 111 and Rubarth found that the majority of adult dog serums contain complement-fixing antibody, suggesting that the infection is widespread and usually symptomless. This inference is substantiated by the isolation of the virus from dog kidney tissue cultures derived from two apparently healthy dogs.²¹

Human infection with the canine virus has been reported,¹⁷⁵ but there is apparently no relation between the human and canine viruses.¹² There is a one-sided antigenic relationship between certain adenoviruses and the canine hepatitis virus in that the latter will fix complement in the presence of antibody to the former, a relationship substantiated by the passive hemagglutination and gel-diffusion techniques.^{29, 63} The canine virus resembles the adenoviruses in being ether-resistant, producing similar CPE, and is of the same order of size.

Murine hepatitis.¹⁴⁰ Naturally occurring murine hepatitis was described in 1951 in England by Gledhill and Andrewes. The original disease was complicated by the presence of *Eperythrozoon coccoides*, but a fulminating infection, lethal in about 1 week, can be produced in the newborn mouse which is characterized by extensive degenerative lesions in the liver. The naturally occurring infection appears to be widespread among infant mice. ¹⁶² The adult mouse is not susceptible to the disease.

The virus is present in the blood, urine, and feces and is infectious by the gastro-intestinal as well as parenteral routes of inoculation. It has not been possible to obtain clear-cut evidence of growth of the virus in the embryonated egg. Inoculation with

relatively large doses of virus results in the appearance of an increased number of monocytes in the chorioallantoic fluid, an effect similar to that observed with human hepatitis virus, but the infection could not be passed in eggs. The virus multiplies in mouse macrophage cultures, but no CPE occurs and the presence of the virus is indicated only by comparative cell counts. More recently the MHV3 strain of the virus has been propagated serially in fetal mouse liver explants on rat-tail reconstituted collagen to produce cytopathic changes, largely in the epithelial outgrowth. ¹⁹¹ It has been found too that the MHV-(Babl D) strain multiplies

in mouse embryo cell cultures and, after passage, in mouse kidney epithelium and L cell culture, giving a cytopathic effect in all three systems. The NCTC 1469 clone of C3H mouse liver, maintained in chicken serum-containing medium, has been found to provide a sensitive assay system, by CPE and plaques, for recently isolated as well as adapted virus strains.

Complement-fixing and neutralizing antibodies have been found in considerable proportions of human serums, 3.5 per cent in children under nine and 21.8 per cent in adults,⁷⁵ suggesting a possible relationship between the human and murine viruses.

The Adenoviruses 26, 66, 179

The viruses known collectively as the adenoviruses were found in 1953, independently and almost simultaneously, by two groups of investigators. Rowe and his colleagues cultured tonsil and adenoid tissue fragments removed at operation as explants in plasma clots in roller tube culture. On continued incubation, a degenerative cytopathology was produced which was transmissible in series in various kinds of tissue cultures, including HeLa cell, fibroblast, and epithelial tissue cultures. The cytopathic changes included the development of acidophilic swollen nuclei which appeared to contain inclusion bodies. The viruses found apparently exist in the form of latent infections in human adenoid or tonsil tissue.

In attempting to isolate viruses associated with acute respiratory disease, diagnosed in part as primary atypical pneumonia, Hilleman and Werner cultured pharyngeal washings from affected individuals in HeLa cell culture. They isolated similar agents producing a degenerative cytopathology in tissue culture and showed that convalescent serums contained significant titers of complement-fixing antibody to the viruses isolated.

Subsequently, similar viruses have been isolated all over the world. They form a group containing a common complement-fixing soluble antigen but fall into distinct serotypes by cross-neutralization tests in tissue culture or by complement-fixation with antiserums prepared in the rabbit¹³³ (see below). This group of viruses was known first as the adenoid degenerative,

or AD, viruses. When their relation to some kinds of pharyngitis and conjunctivitis became clear, they were called the adenopharyngeal-conjunctival, or APC, viruses. Subsequently it was generally agreed that these names would be dropped, and these viruses are now designated the adenoviruses.

Morphology and growth. The adenoviruses are DNA viruses in which the nucleic acid occurs in double-stranded form. The virus particles are 70 to 80 m μ in diameter, and the capsid has cubic icosahedral symmetry and is icosahedral in form. There are 252 capsomeres and no enveloping membrane. These viruses are ether-resistant, stable to acidity, and heat sensitive.

They may be grown in a variety of cell cultures, of which the HeLa line is one of the most susceptible and widely used. They grow within the nucleus of the host cell, and the virus particles may occur in orderly arrays to give a crystalline-like appearance to the inclusion bodies. The CPE is characterized by a rounding and aggregation of the cells.

The human adenoviruses do not show hemadsorption, but some strains produce hemagglutinin and some RDE. They may be conveniently divided into four hemagglutinin (HA) groups on the basis of agglutination of rhesus and rat erythrocytes which parallel immunological groupings. HA group 1 agglutinates rhesus but not rat cells and does not produce RDE; HA group 2 agglutinates rat cells, some serotypes agglutinating rhesus cells to low titer, and produces RDE; HA group 3 gives incomplete

agglutination of rat cells and produces RDE; and HA group 4 does not agglutinate either rat or rhesus cells and does not produce RDE.

In addition to the human adenovirus types, these viruses are also found in lower animals, and occur as simian, bovine, canine, murine, and avian types. These do not appear to be pathogenic for man, nor are human types pathogenic for lower animals. Of these, one of the avian types, GAL (Gallus-adeno-like) has been frequently used for experimental purposes. The types found in lower animals, with the exception of GAL, often show unilateral serological cross-reactions with one or another of the human types.

Antigenic structure. At least two kinds of antigens are present in these viruses which may be analogous to those found in the influenza and related viruses. One is the soluble, complement-fixing antigen which is associated with a smaller particle size, 25 to 40 m μ , than that of the virus particle, and is separable from infectivity. This antigen is group-specific, and gives cross-reactions between the serotypes.

Serotypes within the group are differentiable by neutralization tests carried out in tissue culture, and at least some are also separable by complement fixation, using antiserums prepared in the rabbit by im-

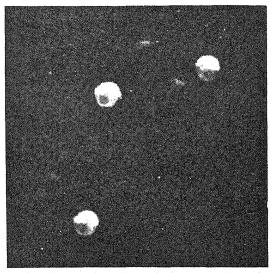
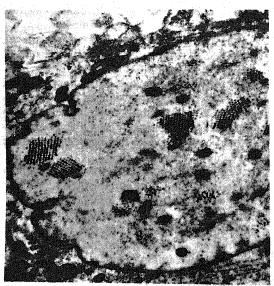


Figure 290. Electron micrograph of shadow-cast preparation of purified adenovirus. Note the dense center of the virus particles. \times 17,000. (Touismis and Hilleman.)

munization with virus grown in tissue culture immunologically heterologous to that used in the preparation of complement-fixing antigens.¹⁵ A total of 28 differentiable serotypes have been described.¹⁵⁷ These types have been given Arabic numbers, and certain of them, types 3, 4, and 7, which have been examined in detail, have been



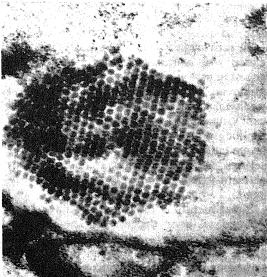


Figure 291. Electron micrographs of ultrathin sections of HeLa cells at 48 hours after infection with adenovirus type 4. Left, single particles and packed arrays of particles are distributed throughout the nucleus. Right, a higher magnification of a similar cell showing the single particles as spherical, ellipsoidal, or polygonal. (Touismis and Hilleman: Virology.)

found to be homogeneous even though the strains were widely different in temporal

and geographical origin.

Pathogenicity. 128, 184 As indicated above, adenoviruses were discovered both as latent infections⁶⁰ in normal persons and in association with respiratory disease. 16, 161 The disease produced by these viruses is an acute infection of the mucous membranes of the respiratory tract and of the eye, with involvement of the submucous lymphadenoid tissues of these areas, including the regional lymph nodes. While it is probable that many of them have the capacity to produce similar disease, there is a definite association of certain serotypes with clinical syndromes, and the evidence that the association is causal is, in some instances, substantially complete. 163 Other serotypes, although found in association with disease, have not been shown to be specific etiological agents. It has been of considerable interest, especially considering their ubiquity, that certain of the adenoviruses, human types, 7, 12, 18, and 31, have been found to be oncogenic in neonatal hamsters. 134

Acute respiratory disease. The adenovirus strains first isolated from military recruits with acute respiratory disease were type 4, and this serotype, together with types 3 and 7, has been found to be closely associated with the disease. The etiological relation of these serotypes to acute respiratory disease is indicated by a number of lines of evidence. In addition to repeated isolation from cases of such disease, rise in specific antibody titer occurs between paired serums from affected persons, and active immunization with polyvalent vaccine has been found to protect 50 to 70 per cent or better of nonimmunes exposed. Such vaccines have been prepared from formalininactivated virus grown in monkey kidney tissue culture. Further, serums from patients with acute respiratory disease, taken during World War II and stored in the interim, have been found to contain antibody to these serotypes, suggesting that the infection was prevalent at that time also. Type 14 has been found to be similarly associated with acute respiratory disease in the Netherlands.

Types 1, 2, and 5, as well as some others, have been recovered repeatedly from tonsils and adenoids removed surgically from persons not ill with respiratory disease, but these types have been found in association with febrile respiratory infections occurring for the most part in infants.

Pharyngoconjunctival fever. 13 This is a febrile disease characterized by pharyngitis and conjunctivitis which was defined as a clinical entity in connection with studies on adenoviruses. It occurs in epidemic form, largely in schoolchildren, and it is estimated that 50 per cent of nonimmunes exposed develop the disease. The conjunctivitis is usually unilateral, nonsuppurative, and does not

result in corneal damage.

It appears to be definitely established that adenovirus type 3 is the specific etiological agent of this disease. The virus has been repeatedly isolated from patients with this disease, both from the throat and from the involved conjunctiva, but not from healthy persons. A rise in specific antibody titer occurs during the disease, and the conjunctival disease has been reproduced in human volunteers by instillation of virus into the eve.

Epidemic keratoconjunctivitis. 180 This is a highly infectious disease characterized by relatively little ocular exudate, the development of round subepithelial opacities in association with the keratitis, and often swelling of the regional lymph nodes. There may also be systemic symptoms, especially headache. The incidence of complications resulting in impairment of vision has been variable. The disease appeared in this country in 1941 and 1942 in epidemic form in shipyard workers on the West Coast and is thought to have been imported from Hawaii.

Adenovirus type 8 has been isolated repeatedly from this condition in Japan, Italy, and Switzerland as well as in the United States. In one series of cases studied.91 62 per cent had neutralizing serum antibody titers of 1:40 or higher and 87 per cent 1:20 or higher, with peak titers as high as 1:160. A four-fold or greater rise in antibody titer between paired serums was found in 16 of 18 cases of the disease. The evidence strongly suggests that this serotype of adenovirus is the etiological agent of this disease.

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Chapter Forty

THE ARBOVIRUSES (YELLOW FEVER, MOSQUITO-BORNE ENCEPHALITIDES, TICKBORNE ENCEPHALITIDES, DENGUE); THE LYMPHOCYTIC CHORIOMENINGITIS VIRUS

The arboviruses, or arthropod-borne viruses, occur primarily in reservoirs of infection in lower animals, including mammals and birds, and in arthropods. The last serve as vectors⁷⁰ to maintain the reservoirs of infection in vertebrates, and to transmit the infection to man, 102 but the human infection is often peripheral to the life history of the virus. 101, 146 These viruses multiply in the arthropod hosts to produce asymptomatic nonfatal infection, and in the case of ticks, but probably not mosquitoes, transovarian transmission of the infection occurs. While transmission to vertebrate hosts, including man, is by the infected mosquito or tick, the same virus is not transmitted by both kinds of arthropod. The great majority of these viruses are transmitted by mosquitoes, and a relatively smaller, and more homogeneous, group is tickborne.171

These viruses thus resemble certain of the rickettsiae, such as those of the spotted fevers, in having a vertebrate-invertebrate biological cycle, ¹⁰⁶ but differ from microorganisms such as the plague bacilli which produce a fatal infection in the insect vector, and from the agents of bacterial or virus infections, such as bacillary dysentery and rabbit myxomatosis, in which the infection is mechanically transmitted by the vector.

The grouping of these viruses as the arboviruses, or arborviruses, rests upon this epidemiological basis, and it is perhaps to be expected that the group includes viruses which appear to be unrelated in other respects.

Very many arthropod-borne viruses have been described, a number commonly given is 125, some having been found in association with disease but others in arthropods with or without serological evidence of vertebrate host infection and are of unknown pathogenicity. These viruses are found in various parts of the world, and many have been given place names of a somewhat exotic character. As a group they are not well known—present information concerning them is summarized by Mussgay¹⁰⁶—and generalizations as to their nature are based upon incomplete evidence.

They appear to be ether-sensitive, heat-labile (56° C.), acid-sensitive RNA viruses, at least some of which have cubic symmetry and probably single-stranded nucleic acid. The capsid is surrounded by an envelope, and most are 30 to 50 m μ in diameter, but some have been reported to be larger, 70 to 130 m μ . Many are cultivable in the chick embryo, in which a fatal infection is produced, and in various kinds of cell cultures. ⁶³

of which hamster kidney and chick embryo cells are the most generally useful. When CPE is produced, it is of a degenerative character, but may be incomplete and give rise to a carrier state. These viruses are pathogenic for the suckling mouse, and some for the adult mouse, producing encephalitis on intracerebral inoculation. In vertebrate hosts, the consequences of infection range from inapparent disease to systemic infection such as hemorrhagic fever with renal complications, yellow fever, and encephalitis which may be highly fatal.

Many of these viruses, though not all, are hemagglutinating for goose and neonatal chick cells. The specificity of the hemagglutinin-inhibiting (HI) antibody has been particularly useful in the immunological characterization of these viruses, allowing their separation into four groups designated A, B, C, and Bunyamwera, but leaving a

residuum of viruses which either do not form hemagglutinin, or whose hemagglutinin is antigenically related to few if any of the other known viruses. Of the established groups, group A includes the equine encephalitides, and group B includes yellow fever, Japanese B and St. Louis encephalitides, and the tickborne encephalitides. Further subdivision may be made on the basis of the specificity of complement-fixing and neutralizing antibodies, but there may be cross-reactions between members of different HI groups. Immunological characterization has, however, served to show the close relationship, or even substantial identity, of arboviruses found in widely separated geographical areas.

Here the more important and representative viruses will be considered individually, followed by a summary of their known interrelationships.

The Encephalitis and Related Viruses of Group A

The better known of the viruses of this group are those of the equine encephalitides and certain viruses found largely in Africa and producing dengue-like disease. There are a number of other viruses of this serological group which are not described here, including the Aura, Bebaru, Getah, Highlands I, Middleburg, Ndumu, Pixuna, Una, and Uruma viruses.

While "sleeping sickness," which may be of bacterial or protozoan etiology, has long been known to occur as sporadic cases and in epidemic form sometimes associated with other disease, the first encephalitis of probable viral etiology to be described with sufficient accuracy for tentative identification was encephalitis lethargica or von Economo's disease. This disease occurred in Roumania in 1915 and in France in 1916, was described by von Economo in Vienna in 1917, and apparently spread to this country in 1918. Epidemics occured in various parts of the world until 1926 but not thereafter. The disease was not of bacterial etiology, but no causative virus was isolated, and the disease has only an uncertain clinical identity. If it was a definite entity, it is not known why it failed to occur after 1926.

Study of the viral encephalitides began in the early 1930's with the isolation of a variety of viruses causing, or potentially capable of causing, encephalitis. The neurotropic character of some of these is marked, but in others it is latent, so that involvement of the central nervous system is relatively rare in the naturally-occurring disease and may be demonstrable only under experimental conditions as in the case of yellow fever virus. Certain of these viruses tend to form a group, related to some degree by antigenic character as well as by spread by arthropod vectors and their occurrence as natural infections of lower animals, and are set apart from other viruses, such as those of herpes simplex, mumps, and measles, and from the psittacosis group of organisms, all of which may at times infect the central nervous system.

EQUINE ENCEPHALITIS VIRUSES

Encephalitis of horses and mules occurring as summer epizootics in North America has been known for many years. ¹⁴¹ A virus was recovered by Meyer and his associates from the brains of horses during an epidemic of the disease in California in 1930 and was found in the blood and central nervous system of human cases of the disease by Howitt in 1938. A similar equine disease occurs in

the eastern part of the United States and Canada, and a virus was isolated from infected animals in 1933 by TenBroeck and Merrill during an epizootic occurring in the central Atlantic Coast states, and from the central nervous system of human cases of the disease in 1938 by Fothergill and his co-workers. A third kind of encephalitis of horses and mules occurs in the northern portion of South America, and is known as Venezuelan equine encephalitis. The causative virus was recovered from infected animals in 1938, laboratory infections in man were reported in 1943, and naturally occurring human disease in the following year. The three viruses are similar in many respects but are distinct antigenically and differ in other ways. The North American viruses have been intensively studied and are somewhat better known than the Venezuelan virus.

WESTERN EQUINE ENCEPHALITIS

The western equine encephalitis or WEE, virus is about 50 mu in diameter as indicated by sedimentation rates, although filtration experiments give a smaller value, 20 to 30 mu. Chemical analysis of purified preparations has shown about 50 per cent lipid, 4 per cent carbohydrate, and the remainder of the ribose type of nucleoprotein, and the virus appears to be a lipid-nucleoprotein complex. It is relatively stable to heat, for 10 minutes at 60° C., and to disinfectants such as ether, phenol, mercuric chloride, and phenyl mercuric borate, and is inactivated by 0.4 per cent formalin after two to four days. The last accounts for accidental infections which have occurred following the inoculation of formalin-inactivated experimental vaccines. It is readily cultivable in the embryonated egg and chick embryo by all routes of inoculation to produce a characteristically rapidly fatal infection of the embryo with generalized hemorrhage, thrombosis and necrosis, and death within 24 hours. It also grows in a variety of tissue cultures,9,75 and in both these and egg cultures large amounts of virus are produced.

The disease in man. The incubation period is from less than one to as long as three weeks, and the human disease varies from an almost symptomless or abortive form to an acute disease in which the patient be-

comes comatose within 24 hours. The disease is essentially a meningoencephalitis with rarely any involvement of the medulla or spinal cord. In the typical case the prodromal signs and symptoms may include headache, drowsiness, and fever. Symptoms of central nervous system involvement may also include tremor, convulsions, mental confusion, and amnesia, but paralysis is not common, occurring in perhaps 15 per cent of cases. The acute phase of the disease lasts seven to 10 days; recovery is usually uneventful and complete. In California in 1952, a peak year, 729 cases of encephalitis occurred, of which almost half were WEE infections, and the case fatality rate in that outbreak was 7 per cent. Serological evidence indicates, however, that many infections with this virus never result in clinical encephalitis, and many others are subclinical.

Experimental infections. The experimental animal of choice is the mouse, which may be infected by the intranasal, intraperitoneal, or intracerebral route. Following intraperitoneal inoculation, the virus appears in the blood, infects the nasal mucosa, and reaches the central nervous system via the olfactory nerves. Within a few days the animals show signs of meningoencephalitis. such as spastic muscular contractions and paralysis, followed by prostration and death, and the disease is similar to that in man. The infection of other animals, including rabbits, guinea pigs, and a number of birds, by the intracutaneous route results in an inapparent infection with viremia to give the kind of infection that probably occurs in animal reservoirs of the virus.

Immunity. Both complement-fixing and neutralizing antibodies are produced as a result of infection and are associated with a solid immunity. Antibody appears early in the infection, usually within a week of onset, and a rise in titer between paired serums is diagnostic. The complementfixing antibody usually disappears within a year, but the neutralizing antibody persists for longer periods and is the one titrated in serological surveys. Experimental animals may be immunized by the inoculation of formalin-inactivated mouse brain antigen, and in such animals the immunity to challenge inoculation is correlated with the titer of neutralizing antibody in the serum. Mice may be effectively protected against both intraperitoneal and intracerebral inoculation, but an abortive infection is produced

in the immunized guinea pig on intracerebral challenge. Mouse brain and chick embryo vaccines have not been particularly successful.⁹¹

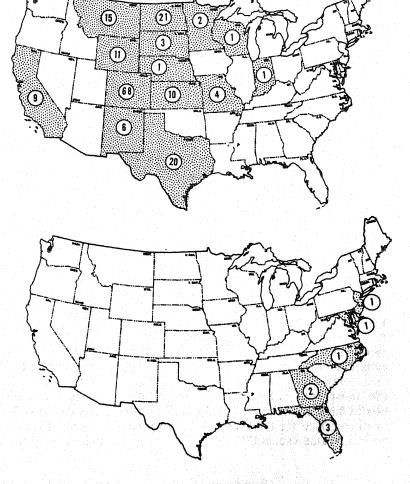
Naturally acquired infection is primarily an infection of wild birds which is transmitted by mosquitoes, and, in the natural history of the virus, infection of horses and man is incidental. The infection occurs west of the Mississippi River, but both serological evidence and isolation of the virus have shown that it is also present in some areas along the Atlantic coast, including New England, 60 and on the Gulf Coast.

Hosts. The virus has been recovered from about 20 species of wild birds and six species of mammals, and there is serological evidence of infection in more than 73 species of wild birds and most common domestic birds and mammals. ⁷⁹ It has been shown experimentally that the level of viremia is considerably higher in infected wild birds

than in domestic birds or mammals, and it may be inferred that mosquitoes may be more readily infected by feeding on the former. While this and other evidence leaves little doubt that wild birds are the important natural reservoir of infection, 115 and that infection in mammals and domestic birds does not play a significant part in the transmission cycle, it is not clear which species of wild birds are of primary importance and which are only secondary. It has been found also that garter snakes (Thamnophis sp.) may be infected, by inoculation or by infected C. tarsalis, the infection remaining latent during winter hibernation, and then becoming activated in the spring to levels of viremia sufficient to infect mosquitoes. 42, 153

Vectors. WEE virus has been isolated from 12 or more species of mosquitoes, from several species of bird mites, and from the assassin bug, Triatoma. Of these, it

Figure 292. The geographic distribution of western (top) and eastern (bottom) equine encephalitis as indicated by the number of cases reported in 1965. The numbers in circles are the numbers of cases reported in the indicated states. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare.)



seems clear that Culex tarsalis is the primary vector of the infection, both in its natural transmission cycle and in the endemicity of the disease in man and horses in the western part of the country. The conclusion is based on detailed consideration of factors such as population density and blood-feeding habits as well as the consistent association of this mosquito with epidemics of the disease in mammals. WEE exists, however, in the absence of C. tarsalis, as in the endemic areas on the Atlantic and Gulf coasts, and has been isolated in those regions from species of Culex, Aedes, and Culiseta. These or other vectors are sufficient to maintain the infection at a relatively low endemic level, with transmission to both birds and mammals, but the relative unimportance of WEE in these areas is apparently due to the absence of an efficient vector such as C. tarsalis and possibly also to a degree of cross-immunity with the eastern virus which results in a lower level of viremia when WEE infection occurs in birds or mammals immune to the eastern virus.

EASTERN EQUINE ENCEPHALITIS

Eastern equine encephalitis, or EEE, is a disease similar to WEE but differing in its geographical distribution, arthropod vectors, and host range. It occurs in Atlantic and Gulf Coast states from New Hampshire to Texas and has been found as far west as Wisconsin in this country, in Canada, Mexico, the Caribbean area including parts of Central and South America, and in the Philippines.

The EEE virus is closely similar in size and chemical composition to the WEE virus and also produces rapidly fatal infections in the chick embryo as well as cytopathic changes in a variety of kinds of tissue cultures. Its pathogenicity for experimental animals parallels that of WEE virus, but it has a somewhat wider host range and is able to infect sheep, cats, and hedgehogs. A comparison⁹⁹ of tissue cultures and experimental animals has shown that chick embryo cells in culture are the most sensitive of the tissue cultures and that the suckling mouse is the most susceptible experimental animal host. Chick embryo cell culture of the virus has been used for the preparation of formalin-inactivated vaccine.91

Naturally acquired infection occurs in both horses and man. In the former the disease is substantially identical with that produced by WEE virus, but infections in man are relatively more severe and are characterized by a higher case fatality rate and a tendency to occur in children. A total of 50 human cases of the disease occurred in Massachusetts in small outbreaks in 1938, 1955, and 1956. On analysis³⁵ of these cases it was shown that 46 per cent of the affected individuals were less than three years old and 68 per cent under 10 years of age. Thirtyfour of the 50 cases were fatal, and recoveries were less satisfactory in younger survivors, with mental retardation, epilepsy, and paralysis of varying extent occurring as sequelae. Similarly, of 13 cases of the human disease which occurred in the Dominican Republic in 1948–1949, only one person was over eight years of age, and nine of the patients died.

Epidemiology.^{21, 64, 76} As in WEE, the natural reservoir of infection of EEE is in wild birds, with infection in mammals and domestic birds playing only a minor part in the natural transmission cycle. Infection of man and the horse is incidental and occurs during periods of high prevalence of the infection.

Hosts. A wide variety of wild birds, more than 40 species, have been found to show serological evidence of infection with EEE virus. In a general way, the smaller birds, including grackles, cardinals, and sparrows, show a higher level of viremia on experimental infection than do larger birds, such as herons and pheasants, and also tend to be more numerous. Further, the proportion of birds showing serum antibody is greater in migratory birds which winter south of the United States than in birds wintering in the north, suggesting a transmission cycle in Central or South America in birds found in this country in the summer.⁷⁸

There is evidence of fluctuation in the prevalence of the infection as indicated by the proportion of successful virus isolations and of birds showing the presence of serum antibody. During periods of low prevalence the incidence of virus isolations is less than 1 per cent and the incidence of antibody 15 to 20 per cent in caught wild birds, and the birds involved include the catbird, cardinal, hermit thrush, and yellow-crowned night heron. During periods of high prevalence

of infections, virus has been isolated from as much as 10 per cent of birds examined, and 50 per cent or more show the presence of antibody in the serum. At the same time the virus is found in additional species, such as the English sparrow and pigeon, and infection of horses and man may represent a similar spill-over of infection during periods of high prevalence.

It is of some interest that outbreaks of EEE have occurred on pheasant farms in Connecticut and in New Jersey. Presumably the infection is acquired initially from an arthropod vector, but direct bird-to-bird transmission occurs to give rise to epidemics of the disease. It is unlikely that caged pheasants play any significant part in the maintenance of the infection but may be considered as a sensitive indicator of the presence of the virus in the vicinity. 74, 92, 125

Vectors. EEE virus has been isolated from a number of species of mosquitoes. including Culiseta melanura, Mansonia perturbans, Anopheles crucians, Culex salinarius, Aedes mitchellae, and pooled Culicoides. C. melanura, in which the proportion of isolations has been relatively high, is a fresh-water swamp mosquito which bites birds freely. The distribution of this mosquito, and A. mitchellae and the saltmarsh species of Culicoides, coincides with the endemic areas of EEE virus infection. It is believed that C. melanura is one of the most important vectors in the maintenance of the natural transmission cycle but it seldom bites man or the horse, and it is probable that other vectors are involved. A. sollicitans, for example, has been found to be an excellent experimental vector, but C. pipiens is not. It is possible also that, with a high infection rate in the horse, mechanical horse-to-horse transmission may occur as a consequence of interrupted feeding of various biting insects such as stable flies, deer flies, and mosquitoes, for such mechanical transmission is demonstrable experimentally.

VENEZUELAN EQUINE ENCEPHALITIS¹³⁰

Venezuelan equine encephalitis, or VEE, occurred as an epizootic in horses and mules in Colombia in 1935, and the causative virus

was isolated during a similar epizootic in Venezuela in 1938. The disease has also been observed elsewhere in South America, *i.e.*, in Argentina, Ecuador, and Trinidad, and has been observed in Mexico¹²⁶ and Florida.²²

The infection in man usually results in a relatively mild, influenza-like disease with headache and fever, gastrointestinal disturbance, myalgia, and lethargy, with little or no indication of central nervous system involvement. Such cases occur as a result of naturally acquired infection, as in Colombia in 1952¹²⁴ and have occurred repeatedly as a result of laboratory infection. The disease lasts for three to five days, occasionally longer in more severe cases. Fatal cases are rare, and in two observed in Trinidad the onset was sudden with signs of encephalitis, followed by coma and death. On the other hand, serological evidence indicates that symptomless or subclinical infections in man may be relatively common in endemic areas. In man the virus is present in the blood and also in the nasopharynx, but there is no evidence of man-to-man transmission of the respiratory infection.

VEE virus infects horses and mules but not cattle. It is less pathologic for birds, and occasional birds, especially pigeons and doves, appear to be refractory to experimental infection. In contrast with WEE and EEE viruses, there is a high-level viremia in infected horses but only a low-level viremia in infected birds. It is probable that the reservoir of infection is mammalian rather than avian and that the disease is transmitted by an insect vector; *Mansonia titillans* transmits the infection under experimental conditions, but the vector of the naturally-occurring disease is not known.

Little is known also of the physical or chemical character of VEE virus, but it is immunologically distinct from WEE and EEE viruses. The usual laboratory animals are readily infected by peripheral inoculation, producing a high level of viremia and encephalitis, and the most useful experimental animals have been the mouse and the guinea pig. The virus grows profusely in the chick embryo and embryonated egg and is cultivable in tissue cultures of human uterus, HeLa cells, and guinea pig and hamster kidney cells.^{39, 75} Experimental animals may be immunized with formalin-inactivated chick embryo virus, and VEE vaccine has

been combined with WEE and EEE vaccines for the immunization of laboratory personnel. A variant, avirulent by other than the intracerebral route, which is an effective immunizing agent under experimental conditions has been described.61

SINDBIS VIRUS

This virus was isolated in 1955 from C. univittatus, and once from a hooded crow, in the Sindbis district in northern Egypt by the intracerebral inoculation of infant mice. It has also been found in India. 129 A number of animals may be infected experimentally. including chickens, horses, doves, and crows, to produce viremia without symptoms, but infant mice show paralysis and die in two or three days. The virus may be grown in the embryonated egg by inoculation of the yolk sac, and the embryos die in one to three days. It may also be grown in chicken embryonic tissue culture with marked cytopathic effects apparent in the fibroblast outgrowth, but no cytopathic effects are produced in HeLa cell or human embryonic skin-muscle tissue culture.38 The virus is 40 to 48 mu in diameter and thus intermediate in size in relation to the viruses described above. It is antigenically distinct.

CHIKUNGUNYA VIRUS

Chikungunya is a dengue-like disease which occurred as an outbreak of infection in the Newala district of Tanganyika in 1953. The disease in man appears to be a clinical variant of dengue in which the headache is lacking, but the eyeballs are tender and there are severe joint pains. In fact, this virus has been found, together with dengue viruses, in epidemics of dengue fever. In a study of the etiology of the disease, Ross¹¹⁹ isolated two viruses - the one Chikungunya virus, which is the etiological agent of the disease, and the other Makonde virus, named after the region (Makonde plateau) in which it was found-whose presence was fortuitous. The two viruses are immunologically unrelated. They also differ in their pathogenicity for the mouse in that the median incubation period of Chikungunya virus infection following intracerebral inoculation was four days, and nine days in the case of Makonde virus; the former killed in four to seven days while the latter required 14 days or longer.

The Chikungunya virus was isolated from the blood of patients with febrile disease and also from A. aegypti, and neutralizing antibody was found in the serum of convalescents. Rabbits and guinea pigs were apparently not susceptible to infection. Although it is a member of group A, this virus gives a slight cross-reaction in the neutralization test with dengue type 1, but not with type 2.

SEMLIKI-MAYARO VIRUS

SEMLIKI FOREST VIRUS

This virus was isolated in 1942 from A. abnormalis in the Semliki Forest of western Uganda by Smithburn and Haddow. 134 It is pathogenic for the mouse by peripheral as well as by intracerebral routes of inoculation and produces fatal infections in guinea pigs, rabbits, and rhesus monkeys on intracerebral inoculation. The brain lesions in infected animals are similar to those produced by the equine encephalomyelitis viruses. The embryonated egg may be infected by various routes of inoculation, including the chorioallantoic membrane, and the infection is uniformly fatal to the embryo. The titer of virus in the body of the embryo is as high as or higher than in the brain, generalized congestion and localized hemorrhages are found in both the brain and body, and the virus appears to be more pantropic than neurotropic in the embryo.

The virus is 30 to 40 m μ in diameter.^{23, 24} It is antigenically distinct and the Mayaro virus found in Trinidad is closely related to it. The Kumba virus, isolated from Eratmapodites mosquitoes in the Kumba region of the British Cameroons in West Africa has been found to be antigenically identical with it. While the Semliki and Kumba viruses have been found only in mosquitoes, the pathogenicity of the virus for man is shown by the occurrence of human disease in Trinidad and possibly elsewhere in South America, and it is apparent that the infection is widespread in the tropical regions of both continents.

MAYARO VIRUS

A febrile disease of viral etiology occurred as an outbreak in Mayaro county, Trinidad, and Casals and Whitman¹⁸ reported the isolation of the causative virus by intracerebral inoculation of sucking mice in 1957. The Mayaro virus is closely related to the Semliki Forest virus found earlier in Africa and, if not identical with it, is a closely related variant. An outbreak of febrile disease of apparently the same etiology occurred in the region of the Guama River in Brazil, and and surveys have shown that antibody to this virus, and the Semliki virus, is widely distributed in the Amazon region.

O'NYONG-NYONG VIRUS

This virus (ONN) was isolated from the blood of patients during a major epidemic of febrile dengue-like disease in Uganda and Kenya, and was also found in anopheline mosquitoes. It appears to be endemic in East Africa, and epidemics of illness occur from time to time. 167 Infected suckling mice show a patchy alopecia and retardation of growth, and mouse passage virus may be propagated in chick embryo fibroblast culture, forming plaques in monolayers. In the plaque-inhibition test the virus shows a one-sided relation to Chikungunya and Semliki Forest viruses.

The Mosquito-borne Viruses of Group B

Of the mosquito-borne viruses of group B, the classic and longest known is that of yellow fever. The viruses of Japanese B encephalitis, dengue fever, and St. Louis encephalitis are well-known and have been studied intensively, while still others are not so well known and/or have been described relatively recently. Some of the last, such as West Nile virus, produce mild disease or inapparent infection as indicated by serological surveys, and are widely distributed in certain areas. In addition to the viruses described here, this subgroup includes the Rio Bravo, Bussuquara, Modoc, Spondweni, Tembusu, and Usutu viruses and the mosquito-borne Wesselsbron virus.

ST. LOUIS ENCEPHALITIS

The disease known as St. Louis encephalitis, or SLE, was first observed in eastern Illinois in 1932 where it was diagnosed as von Economo's disease, and in the following year it occurred in epidemic form in and around St. Louis with an attack rate of 1 per 1000 and a case fatality rate averaging about 20 per cent. The infection occurs in the central states of the midwest and over the entire western half of this country, coinciding with WEE in the latter respect, and has been found in birds and mosquitoes in Trinidad. SLE, WEE, and EEE together

make up the most important arthropod-borne viral encephalitides in this country.

The SLE virus was isolated in 1933 by the intracerebral inoculation of monkeys. In many respects, including a size of 20 to 30 mu, it is closely similar to the WEE and EEE viruses described above, and other encephalitis viruses such as Japanese B encephalitis virus, but it is immunologically distinct and differs in its pathogenicity for various experimental animals. The virus is cultivable in chick embryo and mouse embryo tissue culture and in the volk sac or chorioallantoic membrane of the embryonated egg. Pocks are not produced on the chorioallantoic membrane, but the infected membrane is edematous, with evidence of cell proliferation and focal necrosis. In contrast to the WEE and EEE viruses, SLE virus infection of the embryonated egg does not result in rapid death of the embryo.

The susceptibility of rhesus monkeys to intracerebral inoculation is somewhat irregular, only some strains producing an infection, and the virus tends to be lost on passage; the Cebus monkey appears to be refractory to infection. Infant mice are the most susceptible of the usual experimental animals and may be infected by all routes of inoculation, and adult mice may be infected by intracerebral or intranasal inoculation. Only an inapparent infection is produced in rabbits, guinea pigs, and various birds, and

adult rats, sheep, and ferrets are refractory to infection.

The disease in man. The disease in man is closely similar to WEE, ranging in severity from an abortive type of infection with headache and fever to a severe disease with abrupt onset and symptoms including abdominal and muscular pain, sore throat, conjunctivitis, and signs of neurological disturbance such as ataxia, mental confusion, and occasionally a spastic type of paralysis. The incidence of the disease is relatively high in infants, least in older children, and rises again in the higher age groups. The fulminating type of disease tends to occur in infants, and in a considerable portion, 10 to 40 per cent, of recovered cases there may be evidence of damage to the central nervous system such as mental retardation and hydrocephalus. Recovery in the older groups is usually complete, with sequelae in 5 per cent or less of survivors, but the case fatality rate rises with age, and recovery is often protracted, to several months in severe cases. There is evidence which suggests that inapparent infection may be common.8

Complement-fixing and neutralizing antibodies appear in the serum by the end of the first week of the disease, and a rise in titer between paired serums is diagnostic. Neutralizing antibody, titrated in the mouse, persists for long periods, possibly for life, but complement-fixing antibody declines to insignificant levels within two to three years.

Epidemiology.^{21, 64, 76} Unlike WEE, which occurs in the summer, SLE tends to occur in late summer and early fall. Following an outbreak in St. Louis in 1937, the disease appeared in sporadic form for several years, small urban and suburban outbreaks have occurred since 1954, and an epidemic of over 100 cases occurred in Louisville, Kentucky, in 1956, and one of a similar magnitude in St. Petersburg, Florida, in 1962.⁷

Hosts. The reservoir of SLE virus infection, like that of WEE and EEE, is in birds, and the disease is transmitted by mosquitoes. The virus has been recovered from several species of birds, and serological evidence of infection has been found in 55 species of wild birds and a number of mammals, as well as in most domestic birds and mammals examined. The level of viremia is relatively low, but mosquitoes seem to be more readily infected with this virus than with others in which a high level of viremia is required for infection of the vector. The more important vertebrate hosts appear to be the small perching birds, such as finches and sparrows. and there is also reason to believe that chickens are important epidemic hosts for the virus.

Vectors. SLE contrasts with WEE with respect to arthropod vectors in that the mosquito susceptibility range tends to be

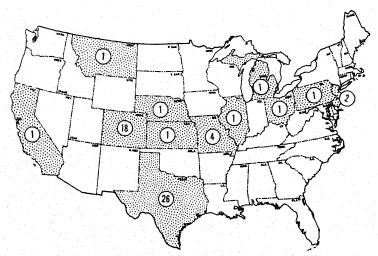


Figure 293. The geographic distribution of St. Louis encephalitis as indicated by the number of cases reported in 1965. The numbers in circles are the numbers of cases reported in the indicated states. (Morbidity and Mortality Weekly Report, Annual Supplement, Vol. 14, No. 53. Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare.)

reversed. WEE and EEE readily infect species of Aedes and Psorophora, but not Culex except C. tarsalis, while SLE is highly infectious for species of Culex but develops poorly in Aedes and Psorophora. These differences are reflected in the epidemiological pattern of the disease, and SLE occurs in two forms, the urban and rural types. The former, in which the disease is found in populated areas of the Midwest where C. tarsalis is scarce or absent, is transmitted to man by mosquitoes of the C. pipiens complex, including C. pipiens. C. quinquefasciatus, C. molestus, and possibly others. In western and rural areas, however, the primary vector appears to be C. tarsalis.

In the rural type of disease in the West, the transmission cycle includes wild, and possibly also domestic, birds as reservoirs of infection, maintained by transmission of the virus by *C. tarsalis*, with occasional infections occurring in man. In more densely populated areas in which *C. tarsalis* is seldom present, the infection is presumably introduced by wild birds and transmitted to local wild and domestic birds, and also to men, primarily by *C. pipiens* and *C. quinquefasciatus*, and outbreaks of the disease in man are associated with relatively dense populations of these mosquitoes.

JAPANESE B ENCEPHALITIS163

Encephalitis of viral etiology occurs with some frequency in various parts of the Far East, and a related form of viral encephalitis has been found in Australia. A closely similar virus appears to be widespread in Africa associated with febrile disease but usually not with symptoms of encephalitis. These viruses are related to SLE and yellow fever viruses on the one hand, and to the Russian spring-summer encephalitis and related viruses on the other.

Encephalitis has occurred in Japan in the late summer for many years and has assumed epidemic form from time to time. The disease has been called Japanese B encephalitis, or JBE, to distinguish it from von Economo's disease which was called Japanese A encephalitis in Japan. An epidemic of over 6000 cases with a 60 per cent case fatality rate occurred in 1924, and out-

breaks occurred every summer to give, in the period 1924 to 1940, a total of approximately 27,000 cases of acute encephalitis. The infection has been found in many parts of the Far East, including China, Russia, Formosa, Thailand, Burma, Malaya, India, the Philippines, and portions of Indonesia, and an epidemic of more than 5500 cases with a 50 per cent case fatality rate occurred in Korea in 1949. Isolation of the virus was reported in 1936 by Japanese workers, Kasahara and his colleagues, and Taniguchi and his colleagues.

The virus is similar to those of the equine encephalitides and SLE in size and properties but has a relatively wider range of pathogenicity for experimental animals. The mouse, monkey, hamster, and young rat are susceptible in that order, the guinea pig is relatively resistant, and rabbits and chickens respond with viremia. Domestic animals, such as the horse, cow, sheep, and goat, may be infected experimentally and show serological evidence of naturally occurring infection in endemic areas, and epidemics of encephalitic disease in horses are often associated with epidemics of the disease in man. In the rhesus monkey an acute encephalomyelitis with relevant symptoms which simulates the human disease is produced following intracerebral or intranasal inoculation, and, in contrast with SLE virus, the infection may be carried in monkey passage. The virus is cultivable in the chick embryo and on the chorioallantoic membrane of the embryonated egg with death of the embryo within 72 hours. It will also grow on a variety of tissue cultures. 6, 83, 116

The disease in man. The disease occurs in man as a symptomless or subclinical infection,52,69 an abortive type of disease in which fever and fleeting signs of central nervous system involvement occur, and as acute meningoencephalomyelitis. In the last there is extensive cortical damage with focal perivascular infiltration, ganglion cell degeneration, and destruction of the Purkinje cells of the cerebellum, and there are lesions in the cord simulating those of poliomyelitis. The onset may be abrupt or insidious, and is characterized by fever, nausea, and disorientation. In the severe disease the neurological symptoms are marked, and include spinal rigidity, tremors, convulsions, and spasticity. The acute phase of the disease may last as long as two weeks, but recovery

is usually complete, and sequelae such as neurological or psychotic changes occur in less than 10 per cent of survivors.

The immune response is evident as the appearance of complement-fixing and neutralizing antibodies in the serum and occurs within one to two weeks after onset of the disease. The neutralizing antibody persists for a long time, and the associated immunity to the disease is thought to be relatively permanent. Complement-fixing antibody may appear in immunes following inoculation of vaccine that will not induce the formation of this antibody to significant titer in the normal individual. A formalin-inactivated chick embryo vaccine has been used for human immunization with suggestive results. 156 Another approach is that of primary inoculation with heterologous but related living virus, the West Nile virus (see below), followed by inoculation with inactivated vaccine of other viruses such as that of JBE.71

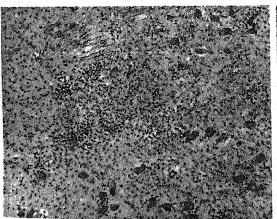
Epidemiology. Natural infection of mammalian hosts other than man with the virus occurs, as indicated by extensive outbreaks among horses and an infection of swine which results in abortion, stillbirth, or affected young, and other domestic animals show serological evidence of infection. Bats may be infected experimentally to give a mosquito-bat-mosquito cycle, but its occurrence in nature is not known. Serum antibody has also been found in a number of

species of Japanese birds, suggesting that there may be avian as well as mammalian reservoirs of infection.⁵⁷

A number of species of culicine mosquitoes, including *C. tritaeniorhynchus* and *C. pipiens* var. pallens, can be infected with the virus, and can transmit the infection under experimental conditions. *C. tritaeniorhynchus* has been found to be naturally infected in Japan, and this and other evidence suggests that this mosquito may be one of the principal vectors of the disease there.⁵⁸

MURRAY VALLEY ENCEPHALITIS

Encephalitis of viral etiology has occurred in Australia from time to time, and the virus appears to be a varient of the JBE virus. Epidemics of acute encephalitis occurred during the summer months between 1917 and 1926, in the first of which the disease occurred largely in small children, with a case fatality rate of 70 per cent, but subsequent epidemics were not as severe. In the severe form the disease was fulminating and resembled JBE in both symptoms and pathology. A virus was isolated from the central nervous system by inoculation of monkeys but unfortunately was lost before its characteristics could be determined, and the disease was called Australian X disease. It has not recurred since 1926.



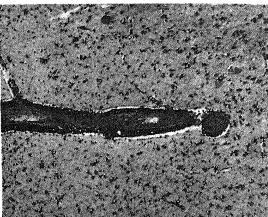


Figure 294. Pathological changes resulting from Japanese encephalitis infection. Left, substantia nigra from a human case; foci of mononuclear cell infiltration are present, and numerous ganglion cells are in a disintegrated state or have disappeared. Hematoxylin and eosin; \times 90. Right, region of superior temporal gyrus from a human case; a vein at the corticomedullary junction is surrounded by a "cuff" of lymphocytes. (Haymaker and Sabin: Arch. Neurol. Psychiat.)

DENGUE 953

In the summer of 1950–1951 an epidemic of encephalitis occurred in the Murray Valley in Victoria, Australia, in which 40 cases were recognized and 17 of these were fatal. A few cases occurred in 1956, and the disease seems to appear only sporadically. It has also been observed in New Guinea, where serological surveys have suggested a patchy distribution of the infection. The disease is called Murray Valley encephalitis, but there is reason to believe that it represents a recrudescence of Australian X disease.

A virus was isolated on the chorioallantoic membrane of the embryonated egg, killing the embryo in two to three days, and adapted to infant mice in which it produced encephalitis, fatal in about a week, on intracerebral inoculation. The virus was found to be closely similar to, but not immunologically identical with, JBE virus.^{1, 133}

Complement-fixing and neutralizing antibodies, the latter demonstrable in the mouse, are produced in response to infection, and surveys in the epidemic area showed that about 5 per cent of persons with no history of the disease had significant titers of complement-fixing antibody. It was found in further studies that antibody is relatively widely distributed throughout eastern Australia, not only in man, but also in horses and a number of species of wild birds. 103 There is circumstantial evidence which suggests that C. annulirostris transmits the disease.96 The disease may, like SLE and the equine encephalitides, exist in a reservoir of infection in wild birds, maintained by mosquito and possibly bird mite transmission. It is transmitted to man and other mammals by mosquitoes.

WEST NILE VIRUS

The isolation of this virus from a native of Uganda with a mild febrile illness was reported by Smithburn in 1940; it was subsequently isolated in Egypt in 1951;¹⁰⁰ and outbreaks of the disease caused by it have been observed in Israel.^{46, 95} In contrast to JBE, the disease is rarely fatal and usually does not take the form of encephalitis. The incubation period of the disease in Israel was two to six days, and it was characterized by an abrupt onset, fever, drowsiness, and

severe frontal headache. A maculopapular rash may develop, more commonly in children, there may be abdominal pain with anorexia and nausea, and there is a general enlargement of the lymph nodes. The acute phase of the disease persists for less than a week, but convalescence may be protracted.

The virus is closely similar to the JBE virus, being of the same order of size and immunologically related to it and to SLE virus. It has been of particular interest in connection with active immunization to infection with closely related viruses such as JBE virus and others. Mice may be infected by the intracerebral route, and the infection is characterized by degenerative changes in the Purkinje cells of the cerebellum in both these animals and in monkeys. The virus grows on the chorioallantoic membrane of the embryonated egg and in a number of kinds of tissue cultures.

Serological evidence indicates that the infection is widespread in Africa, probably as inapparent infections, not only in Uganda and Egypt, but also in South Africa, the Republic of the Congo, and the Sudan. As many as 70 per cent of persons examined in Egypt show significant antibody titer, and the disease has been studied extensively there. The disease occurs largely during the summer and is sporadic rather than epidemic in form. The occurrence of outbreaks of the disease in Israel in 1951 and 1954 may have been due in part to relatively recent introduction of the virus.

The virus has been isolated from birds, pigeons, and the hooded crow in Egypt, and there may be an avian reservoir of infection. Virus has also been found in *C. univittatus* and *C. antennatus*, and *C. pipiens* and *A. aegypti* transmit the disease experimentally. *C. univittatus* is regarded as the most probable important vector of the infection.

DENGUE¹²²

Dengue, or breakbone fever, is an infectious disease transmitted by mosquitoes which is endemic, and sometimes epidemic, in tropical and subtropical climates; it may occasionally occur in temperate zones. Epidemics may be very large; it is estimated

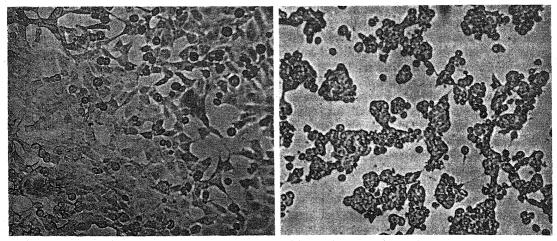


Figure 295. The CPE produced by West Nile virus in HeLa cell culture. Left, uninoculated cell culture; right, four days after inoculation with virus.

that there were between one and two million cases of the disease in the southern states in this country in the epidemic of 1922, more than a million cases occurred in Greece in 1927–1928, and a greater number in Japanese port cities from 1942 to 1945, with one-third to one-half the population of Osaka affected in 1944. It was first studied in the Philippines by medical officers of the United States Army, who established the viral nature of the etiological agent and its transmission by A. aegypti.

Dengue also occurs in a more serious form as a kind of hemorrhagic fever which has occurred in epidemic form in the Philippines and in various parts of southeast Asia, as in Singapore, Thailand, and Vietnam.

Dengue viruses. The virus is similar in size to the yellow fever and encephalitis viruses described above, possibly slightly smaller, and its size is usually given as 12 to 25 m μ . In electron micrographs dumbbell-shaped particles are observed, but it is not established that these represent virus particles. In the 1950's serotypes were differentiated, first types 1 and 2, which are by now the best known, and subsequently types 3 and 4, which were found in studies on hemorrhagic fever; there may be two additional serotypes, types 5 and 6.

On primary isolation the dengue virus does not produce CPE in monkey kidney cell cultures, but growth is inferred from resistance, increasing with passage, to superinfection with poliovirus, *i.e.*, interference. Following adaptation, usually by intracerebral passage in the mouse, the virus grows with CPE and plaque formation in monkey

kidney tissue cultures,⁴⁵ and mouse-adapted virus may be propagated serially in HeLa cells with CPE¹⁰ and production of hemagglutinin.¹¹ The serotypes are differentiated by neutralizing and complement-fixing antibodies and by HI, and seem to be more closely related to one another than to other members of group B by the last criterion.

Pathogenicity for lower animals. Various monkeys, including species of Cynomolgus, Ceropithecus, and Macacus, and chimpanzees may be infected with the production of antibody, but the infections are symptomless, and even inapparent infections are not produced in the usual experimental animals. Newborn mice, however, may be infected by intracerebral inoculation, with the development of immunity but no symptoms, or minor symptoms, of disease on primary isolation.

On passage the virus may become adapted to mice, giving high titers of virus in the brain, producing motor disturbance and partial flaccid paralysis with or without signs of encephalitis. Adult mice may be infected by intracerebral inoculation of well-adapted strains but are refractory to other routes of inoculation. A number of strains of both types of the virus have been so adapted to mice. Adapted virus may produce fatal paralytic disease in rhesus monkeys, simulating experimental poliomyelitis, following intracerebral inoculation. In general, there is no satisfactory experimental animal host for unmodified virus, and infection experiments have been carried out with human volunteers.

Dengue fever. Experimental studies

have shown that, following intracutaneous inoculation of as little as 10 minimal infecting doses (MID) for man, local erythema and edema develop in three to five days which are associated with local multiplication of the virus. Sabin has used this reaction for immunological studies for, when virus is mixed with appropriate amounts of antiserum prior to inoculation, the reaction does not occur. With the onset of fever, the blood may contain as much as one million MID per ml.

The incubation period is usually five to eight days. The prodromal symptoms include headache, backache, and malaise, followed in six to 12 hours by an abrupt rise in temperature. The acute phase of the disease is characterized by headache, the severe pain in the muscles and joints which has led to the name breakbone fever, abdominal pain, constipation, and anorexia. A maculopapular eruption occurs with variable frequency which appears about the third day of the disease, spreading from the trunk to the extremities and face, and persists for three to four days. The febrile period lasts for five or six days and commonly terminates by crisis. The disease is seldom if ever fatal.

Hemorrhagic fever.⁵¹ The occurrence of disease characterized by severe hemorrhage or shock with 10 to 15 per cent mortality has been known in association with dengue fever epidemics since the turn of the century. The dengue virus etiology of this form of the disease was first recognized in the Philippines in 1956.55 Subsequently it has occurred in epidemic form in Thailand, 53, 56 Malaysia,90 and Vietnam.54 From 1961 to 1965 more than 16.000 cases have occurred in children in Thailand with about 1000 deaths; because of its presence there it is also known as Thai hemorrhagic fever. This kind of disease occurs most frequently in children under 12, and is characterized by capillary damage, visceral lesions especially in the liver, petechiae, blood in vomitus and stools, and shock. It may be associated with any of the serotypes of the virus, and there is epidemiological evidence suggesting that some strains may be more virulent than others.

Other hemorrhagic fevers. Hemorrhagic fever occurring in the Western Hemisphere, in South and Central America, ¹⁴⁸ is of different viral etiology. A virus, designated Junin virus, ¹¹³ was isolated in Argentina during an epidemic in 1958. Hemorrhagic

fever occurred in Bolivia,93 first in 1959, and the viruses isolated were found to be indistinguishable from the Junin virus by complement fixation, but distinct by the neutralization test. The prototype strain of this group is the Carvallo strain, and the viruses as a group are designated the Machupo virus.⁷³ Both have been found in small rodents and. in the case of Junin virus, in mites, but there seems to be no evidence of arthropod transmission of the Bolivian viruses. These are, nevertheless, considered to be arboviruses, but are ungrouped since they are unrelated serologically to the HI groups. Another immunologically related virus, Tacaribe virus, has been isolated from bats in Trinidad, but is not known to be associated with disease in other forms.32

Still another kind of hemorrhagic fever was recognized in Manchuria in 1939 by Japanese workers, and appeared among United Nations military personnel in 1951 in Korea, where approximately 1000 cases of the disease occurred. It was shown by both Japanese and Russian workers that the disease could be transmitted to human volunteers by the parenteral inoculation of blood or urine taken early in the febrile period of the disease, that the causative agent passed bacteria-proof filters, and that convalescent serum is protective. While this evidence is taken to indicate that the disease is of viral etiology, it has not been possible to produce infections in experimental animals nor to culture a virus by the usual methods, and the etiology remains somewhat obscure. The epidemiological character of the disease is consistent with that of an arthropod-borne infection existing as an animal, possibly rodent, reservoir of infection, a conclusion reached by Japanese, Russian, and American workers. 41, 131, 144 This kind of hemorrhagic fever is more widespread than had been originally supposed and occurs across northern Asia and Europe from Korea to Scandinavia.25 It is not to be confused with Crimean or Omsk hemorrhagic fevers, which are tickborne.

Immunity. Recovery from dengue either in man or in those experimental animals which can be infected with the virus, results in a solid immunity and the appearance of complement-fixing and neutralizing antibodies in the serum. Complement-fixing antigen may be prepared from mouse brain, and protective and neutralizing antibodies are demonstrable by challenge of immunes

in the first instance, and by the neutralization test using the skin reaction in man noted above, or in mice, in the second.

The solid immunity is to the homologous serotype and has been shown experimentally to persist for at least 18 months. Convalescents have a considerable degree of initial immunity to the heterologous serotype of virus, but it disappears relatively rapidly. Thus, within two months after recovery, the heterologous immunity probably often suffices to prevent naturally acquired infection. It deteriorates thereafter so that infection between two and nine months after recovery with the heterologous serotype results in a modified form of the disease which is of shorter duration, without rash, and generally less severe. Active immunization with mouse-adapted strains of the virus has given encouraging results. 123, 127

Epidemiology. The animal reservoir of dengue virus appears to be man and various species of monkeys in which viremia occurs even though the infection is symptomless; birds do not seem to be infected. The virus is transmitted by the domestic mosquito, A. aegypti, as noted earlier, and also by A. albopictus, which can exist in the bush or jungle and transmit the infection among primate hosts other than man. Epidemiological observations suggest that A. scutellaris and A. polynesiensis may also be vectors under natural conditions. It may be noted that A. vexans, A. taeniorhynchus, C. pipiens, and anopheline mosquitoes such as Anoph, quadrimaculatus and Anoph. punctipennis are apparently unable to transmit the infection.

Man is infective for the mosquito: i.e., there is viremia, for a period including the last day of the incubation period and three days or more after onset of the disease, and presumably monkeys are similarly infective since viremia is demonstrable experimentally for five to eight days after infection. The extrinsic incubation period is 10 days to two weeks, and the mosquito remains infectious for life, but there is no congenital transmission of the virus. Endemic persistence of the infection depends upon climatic conditions such that mosquitoes may survive throughout the year, together with a sufficient number of newborn mammalian host susceptibles to maintain the transmission cycle.

NTAYA VIRUS

This virus was isolated135 in 1943 from pooled mosquitoes of several genera and species, including Culex and Aedes, collected in the Ntava swamp in western Uganda. The infection was uniformly fatal to mice by intracerebral inoculation after the eleventh passage, but the intranasal and intraperitoneal routes of inoculation were not effective. Infections uniformly fatal to the embryo are produced by inoculation of the volk or amnionic sacs, but embryos die only irregularly following inoculation on the chorioallantoic membrane or allantoic cavity. The virus is neurotropic in the embryo, with higher concentration of the virus in the brain than in other parts of the body. and the gross hemorrhagic lesions are confined to the brain. The virus is 70 to 120 m μ in diameter and antigenically distinct but has been found to give some cross-reaction with Ilheus virus in the complement-fixation reaction. There is no evidence of naturally occurring mammalian infection, and persons living in the vicinity of the swamp did not show serum antibody.

UGANDA S VIRUS

The Uganda S virus was isolated in 1947. in Bwamba county, Uganda, from a pool of mosquitoes containing A. longipalis, A. ingrami, and A. natronius, by intracerebral inoculation in the mouse.29 Hamsters may be infected by the intracerebral route, but the infection is not uniformly fatal. The virus may be grown in the embryonated egg by yolk sac and amnionic sac inoculation, reaching higher concentrations in the brain than in the body of the embryo. The embryos are usually not killed, and the gross lesions are minor, consisting of congestion and some small hemorrhages in the brain. The virus is 15 to 30 m μ in diameter, and antigenically distinct, but gives irregular cross-reactions in the complement-fixation test with some yellow fever virus antigens. Neutralizing antibody has been found in human serums, with 7.7 per cent positives found in Uganda and Tanganyika, and 35 to 50 per cent in Nigeria. On this basis, the virus would seem to be one of the most common in Nigeria. CONTRACTOR OF THE STATE OF THE STATE OF

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ZIKA VIRUS

This virus was isolated in 1947 and 1948. first from the blood of a captive monkey (vellow fever sentinal) in the canopy of the forest near Zika, Entebbe, Uganda, and again from a pool of A. africanus in the same region.30 It was isolated in mice by intracerebral inoculation but is not pathogenic for hamsters or for mice by the intraperitoneal route. It is cultivable in the embryonated egg by inoculation into the volk. amnionic, and allantoic sacs but usually does not kill the embryo: gross lesions are uncommon, and the virus is pantropic rather than neurotropic in the embryo. The virus is 15 to 30 mu in diameter and antigenically distinct.

ILHEUS VIRUS

The Ilheus virus was isolated in 1947 in Brazil by Laemmert and Hughes⁸¹ from pooled Aedes and Psorophora mosquitoes. It is also found in Panama, where birds may be hosts.⁴⁰ It is cultivable on the chorioallantoic membrane of the embryonated egg but disappears from this site relatively rapidly. The embryo is infected following yolk sac inoculation, and the virus is found in highest concentration in the brain. It is highly pathogenic for mice, even when inoculated by other than the intracerebral route. It is 15 to 30 m μ in diameter.

YELLOW FEVER¹⁴³

Yellow fever is an acute disease, occurring in epidemic and endemic form, which was first observed in Central America in the middle of the seventeenth century. It is uncertain whether the original focus of infection was in Africa or the Western Hemisphere, but in the eighteenth and nineteenth centuries the disease was widely distributed in the Caribbean area, including the adjoining coasts of North, Central, and South America. It occurred in epidemic form in more northern cities in this country, including Philadelphia, New York, and Baltimore, and it is estimated that there were at least half a million cases of the disease in this country in the nineteenth century. It occurred in epidemic form in Spain in 1800 with 60,000 deaths and in Rio de Janeiro with more than 20,000 deaths between 1851 and 1883, and there were almost 36,000 deaths from the disease in Havana during the same period.

The occurrence of the disease in American military personnel during the Spanish-American war resulted in the classic studies of the Yellow Fever Commission of the United States Army under Dr. Walter Reed at the beginning of the twentieth century, with evidence of mosquito transmission and the viral nature of the infectious agent. Later work appeared to suggest that the disease is a leptospirosis, but in the late 1920's its viral etiology was firmly established.

The virus, shown to be filtrable by the work of the Commission, is one of the smaller viruses, with a diameter of 30 m μ , and it is readily filtrable through the usual bacteriaproof filters. It is relatively labile, is readily inactivated by heating and by the usual disinfectants, but is preserved in 50 per cent glycerol and in lyophilized form.

The disease in man. The disease is transmitted to man by the bite of infected mosquitoes, and the incubation period was found to be one to three days in early experimental infections. The onset is sudden with headache, backache, and rigor, and the temperature rises rapidly to reach a maximum within 48 hours. In the first, or congestive, stage of the disease there is nausea and vomiting, the face is flushed, and a tendency to hemorrhage becomes apparent. After three or four days the temperature falls, then rises again, and the second stage of the disease is characterized by venous stasis and a marked tendency to hemorrhage; prostration occurs, jaundice appears but is not marked, and the kidneys are involved and there is albuminuria. Death occurs by the sixth or seventh day, seldom after the tenth day, or recovery is rapid and uneventful. Many infections are mild or almost symptomless. and the fatality rate is not known with precision; it is estimated to be about 5 per cent.

The disease is one of the hematopoietic system, there is little inflammatory reaction, and the tissue changes are degenerative in nature. These occur in the liver, kidney, and heart, and there is evidence of hemorrhage. The characteristic, and pathognomonic, lesion is a hyaline type of necrosis in the

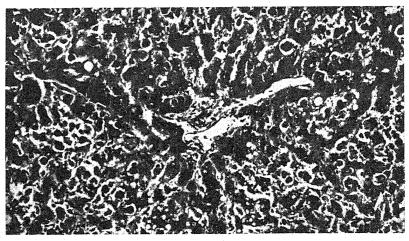


Figure 296. Section of liver from a human case of yellow fever. Midzonal necrosis is apparent. Hematoxylin and eosin; reduced from x 235. (Theiler.)

midzone of the liver, and the affected cells are known as Councilman bodies. Fatty degeneration occurs in the kidney tubules but is not distinctive, the spleen is hyperemic and shows degenerative changes, and degenerative changes are found in the heart.

Experimental infections. The human disease is closely reproduced in the rhesus monkey,4 and a number of species of monkeys are susceptible to yellow fever; in fact, natural infection appears to be widespread among primates in certain areas (see below). The neurotropic tendencies of the virus are demonstrable in the monkey by intracerebral inoculation, and simultaneous protection of the viscera by antiserum, to produce an encephalitis. The rabbit and rat are resistant to experimental infection, but encephalitis is produced in the guinea pig on intracerebral inoculation. The infant mouse is susceptible to parenteral inoculation by any route; the adult mouse is susceptible to intracerebral inoculation but is seldom infected by intraperitoneal inoculation. Some strains of mice are considerably more susceptible than others. Passage by intracerebral inoculation in the mouse results in adaptation to the mouse brain, and such strains lose their viscerotropic tendencies and become less virulent for the monkey.

Tissue culture. Neurotropic virus is cultivable without difficulty in the Maitland type of tissue culture of chick embryo which includes nerve tissue, but the unadapted pantropic strains may be difficult to grow. The Asibi strain of yellow fever virus was grown first in mouse embryo tissue culture, then chick embryo tissue culture, and after prolonged passage a variant appeared which

had lost both viscerotropic and neurotropic tendencies. This variant is the 17D strain, now widely used as an immunizing agent.

This strain will form plaques in chick embryo cell monolayers, and the system may be used to titrate antibody. ¹¹¹ It grows in HeLa and KB cell cultures with a cytopathic effect characterized by rounding up of the cells and detachment from the glass. ⁵⁰ It may also be grown in various kinds of primary cell cultures. ⁶⁸ In human embryonic intestine, human amnion or Chang's liver cell culture the virus is demonstrable by the fluorescent antibody technique as a perinuclear fluorescence. ²⁸

Epidemiology. Yellow fever occurs in animal reservoirs of infection and in infected mosquitoes and is transmitted to the vertebrate host by the insect vector. The epidemiological character of the disease is determined by the species and habitat of both vertebrate and invertebrate hosts and occurs in two epidemiological types, the one urban yellow fever, and the other jungle yellow fever.

Urban yellow fever. It was established at the turn of the century that the disease is transmitted to man by Aedes aegypti, and in the urban type of disease the life history of the virus consists of a man-mosquito-man cycle in which the mosquito is infected by man, requires 10 to 15 days at tropical temperatures to become infectious, and remains infected for life. This mosquito is domestic, living largely in and around human habitation, the foci of infection are in urban areas, and the disease is one of communities. Since man-to-man infection does not occur under natural conditions,

control of the vector in urban areas allows control of the human disease. Control programs directed toward this end have been highly successful, and the disease appeared to have been substantially eradicated in the Western Hemisphere by the middle 1920's.

By the late 1920's vellow fever appeared in small communities and rural areas in South America, and it was apparent that the man-mosquito-man cycle may persist in areas in which there are small groups of people in frequent communication with each other. Control consists of extension of mosquito control measures to such small communities and even to rural areas.

Jungle yellow fever. In the early 1930's vellow fever occurred in Brazil in the absence of A. aegypti, and the infection has been found to persist in reservoirs of infection in lower animals and to be transmitted by mosquitoes other than A. aegypti. This is sylvan or jungle yellow fever in which human infection is peripheral to the life history of the virus. The human disease is found in man coming in contact with forests or the edge of forests and tends to occur in adult males whose work takes them to such infected areas. This is in contrast with urban yellow fever in which all ages and both sexes are exposed to equal risk. Infection of man, followed by infection of A. aegypti may, of course, initiate the man-mosquitoman transmission cycle characteristic of urban yellow fever. The endemic foci of jungle yellow fever are in South America, Africa, and Central America, and differ somewhat from one to another of these areas.

South America. The area of endemic infection in South America, as indicated by the occurrence of antibody in young children as well as adults to show the recent or present occurrence of the disease, covers much of the tropical zone, including the Amazon and Orinoco regions, Colombia, the Guianas, and Panama. Within this area forest monkeys appear to be the most important vertebrate host, although in some areas where monkeys are few, marsupials such as the opossum show serological evidence of infection. Some species of monkeys, especially the howler monkey, Alouatta, suffer from epizootics of the disease which are fatal to large numbers of the animals. On the basis of the presence of antibody in the serum of wild caught specimens, the principal genera serving as vertebrate hosts seem to be Alouatta, Cebus, and Ateles, and others may be involved also.

Yellow fever virus has been isolated from forest mosquitoes. Haemagogus capricornii, H. spegazzini, and the subspecies H. spegazzini falco. Other species of the same genus, and one of the genus Trichoprosopon, transmit the disease experimentally and may be regarded as possible vectors. Species of Haemagogus are found over the entire area in South America in which the infection is endemic and are no doubt an important element in the transmission cycle. They "forest-canopy," tree-hole breeding mosquitoes and are found in the tops of trees, rarely coming to the ground except at the edge of the forest.

Africa. 145 In Africa A. aegypti is widely distributed, and both the urban and jungle types of vellow fever occur. As in South America, serological evidence indicates that the vertebrate hosts in the latter form of the disease are forest primates of many species, including lemurs. A. africanus is found in many parts of central Africa and is known to be an important factor in the maintenance of vellow fever virus in the forest of Uganda. Like Haemagogus in South America, it is an arboreal mosquito, and it readily bites monkeys, but does not attack man. A. simpsoni, which bites both monkeys and man, is also present, and is thought to be the mosquito responsible for transmission of the infection from monkeys to man at the edge of the forest.

Central America. 15, 157 The available evidence shows that the jungle type of yellow fever has been moving north. In 1954, after 25 years with no record of yellow fever, cases of the disease occurred in man in Port-of-Spain, Trinidad. Mortality in monkeys was high during that year, and yellow fever virus was isolated from sick monkeys. The virus was also recovered from Haemagogus mosquitoes, and some of the cases of the disease in man were probably acquired from A. aegypti which had not been kept under control, i.e., the urban type of the disease. Similarly, in 1948 cases of yellow fever were reported in eastern Panama for the first time since 1905. It subsequently moved as a wave in a general northwesterly direction into central and western Panama. Costa Rica, and Nicaragua, and reached Honduras in 1954. In early 1956 yellow fever appeared in monkeys in Guatemala, and the virus was isolated from two species of mosquitoes, H. equinis and Sabethes chloropterus. The virus has also been recovered from other Central American mosquitoes, including *H. spegazzini falco*, *H. lucifer*, and an anopheline, but their status as vectors is uncertain.

In contrast to the situation in South America, where Cebus monkeys are in the majority and show little mortality from naturally-acquired yellow fever, the howler (Alouatta) and spider (Ateles) monkeys are predominant in Central America and show a high mortality from yellow fever.

Immunity. Recovery from yellow fever results in a solid immunity, often said to persist for life, and strains of the virus, even from widely separated areas, are of a single antigenic type. The antibody response is demonstrable as HI, complement-fixing, and neutralizing antibodies and the three are not identical. The complement-fixing antibody appears later than neutralizing or HI antibody during infection and may not occur to significant titer in mild infections. It disappears within a few months, and is usually not formed in response to prophylactic inoculation with the 17D strain of virus, and therefore has some value in detecting recent infections. 151

The neutralizing antibody appears quite early in the disease, often by the fifth day, and persists more or less indefinitely. The titration of neutralizing antibody is used for serological surveys for the prevalence of infection. It has been carried out in various ways, and at the present time the method most commonly used consists of mixing varying dilutions of the serum with a stand-

ard amount of virus and testing for residual activity by intracerebral inoculation of adult mice or intraperitoneal inoculation of infant mice.

Prophylaxis. While vector control suffices to control urban yellow fever, it is ineffective in the control of the jungle type of the disease, and in this and certain other circumstances active prophylactic immunization is required. It was early apparent that inactivated virus does not induce an effective immune response,²⁷ and living attenuated virus is used. Two kinds of virus are used for this purpose, one a mouse-adapted neurotropic strain which is used largely by the French in Africa, and the other the 17D strain described above.

The former is employed as a lyophilized preparation which is suspended in a solution of gum arabic just before use and applied by scarification. As used by the French workers in Africa, it is often combined with vaccinia virus. Vaccine is prepared from the 17D strain as lyophilized chick embryo tissue, reconstituted in saline, and inoculated subcutaneously in doses containing 500 mouse MLD. The incidence of untoward reactions is low, perhaps 5 per cent, and such reactions are usually mild. The neurotropic strain appears to give a somewhat better immune response than the 17D strain as measured by neutralizing antibody titer, and in either case the immunity so produced is effective for considerable periods of time; persisting antibody titers have been found in immunized persons as long as 17 to 19 years after primary inoculation. 49, 118

The Tickborne Viruses of Group B

The arboviruses transmitted by various species of ticks are set apart epidemiologically from the mosquito-borne viruses. Some fall into group B as a relatively homogeneous subgroup, the Russian spring-summer encephalitis complex, within which the component viruses are more closely related to one another than to the other members of group B. In addition to those considered here, the tickborne viruses of this subgroup include the Entebbe, Langat, and Negishi viruses. Other tickborne viruses are unrelated to group B. Of these, some fall into two small groups of antigenically related viruses, that made up of the Chenunda. Nyamanini, and Quaranfil viruses and that

including the Kemerovo, Koliba, Lipovnik, and Tribec viruses. The miscellany of sero-logically unrelated viruses includes the Crimean hemorrhagic fever, Kaisodi, Nairobi sheep disease, Silverwater, Thogoto, Wad Medani, and tickborne Witwatersrand viruses.

RUSSIAN SPRING-SUMMER ENCEPHALITIS^{138, 147}

Russian spring-summer encephalitis, or RSSE (Russian Far East encephalitis, Russian forest-spring encephalitis, Russian tickborne encephalitis, Russian vernal en-

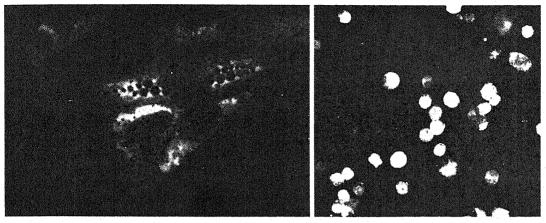


Figure 297. Fluorescent antibody stained tickborne encephalitis virus in chick embryo cell culture. *Left*, smear of cells from culture showing immunofluorescence in some of the cells. × 800. *Right*, fluorescent virus in the cytoplasma of infected cells. × 200. (Albrecht and Kožuch, Bull. Wld. Hlth. Org.)

cephalitis, Russian endemic encephalitis), is not to be confused with Russian autumnal encephalitis, which has been shown to be Japanese B encephalitis. It was observed in 1937 in the far eastern provinces of the Soviet Union and has subsequently been found in other parts of Russia, in Central Europe, including Czechoslovakia, Bulgaria, Poland, Yugoslavia, and Austria, and in Germany.

The virus is 15 to 25 m μ in diameter and may be grown on the allantoic membrane of the embryonated egg and in cultures of human embryo muscle tissue with the production of cytopathology. The mouse may be infected by subcutaneous as well as intracerebral inoculation and is extremely susceptible to mouse-adapted virus. The virus is pathogenic for sheep and rhesus monkeys, but not for guinea pigs or rabbits. A number of wild birds and rodents may be infected experimentally to give a symptomless viremia, and wild rodents have been found to be naturally infected.

The disease in man. The incubation peroid is 10 to 14 days, and the febrile disease in man is characterized by signs and symptoms of a meningoencephalitis or polioencephalitis. It varies in severity from an abortive type of perhaps a week's duration terminating in complete recovery, through a moderately severe, and more prolonged, type of encephalitis with a 20 per cent case fatality rate, and often neurological sequelae in survivors, to a severe fulminating type of meningoencephalitis with death occurring within a week. The overall case fatality rate

is about 30 per cent, but there is a high incidence of antibody in human populations in endemic areas, and it is probable that many infections are symptomless or subclinical.

The gray matter of both brain and cord is involved, with neuronal necrosis and neuronophagia and more marked glial and mesodermal reactions than occur in the other viral encephalitides. The disease is characterized by evidence of bulbar involvement and a peripheral type of flaccid paralysis, most often of the brachial and cervical muscles, which may be residual. In fatal cases parenchymatous degenerative changes may be found in the heart, kidney, and liver. The virus is present in the blood and spinal fluid during the course of the disease and may be isolated from the brain at autopsy.

Immunity. An immune response is evident two to three weeks after onset of the disease as complement-fixing and neutralizing antibodies in the serum. The solid immunity to reinfection is long lasting and associated with the presence of neutralizing serum antibody which reaches peak titer in two to three months. Russian workers have used formalin-inactivated brain and chick embryo vaccines for active immunization and recommend the administration of hyperimmune goat serum on exposure of susceptible persons.

Epidemiology. The first cases of this disease to be reported were seen in persons whose work took them into forested regions, but it is now clear that the distribution of the disease is less restricted, and it has oc-

curred, sometimes in high incidence, in populated areas with greater numbers of infections in children. As noted above, the incidence of serum antibody may be relatively high in endemic areas, and human infection is doubtless more common than the number of clinical cases of the disease would indicate. Whether or not human infection may, under some circumstances, constitute a significant element of the reservoir of infection is open to question. The infection occurs in goats, and these, and possibly other, domestic animals may be a source of human infection. Human infection may occur by the alimentary route,87 and infection, especially in urban areas, from the milk of infected goats undoubtedly contributes both to human disease and to inapparent but immunizing infections. 110 It is generally believed that the significant reservoir of infection is in wild mammals and birds⁵⁹ in which viremia occurs, but the infection is otherwise essentially symptomless.

The infection is transmitted by ticks, of which *Ixodes persulcatus* is regarded as the principal vector. The infection is congenital in these ticks, infection rates as high as 40 per cent have been found in certain small areas, and there is little doubt that there is an arthropod reservoir of infection. Natural infection of other ticks, *Haemaphysalis concinna*, *Dermacentor sylvarum*, and so-called gamasid ticks or mites found in bird and rodent nests, has been found also.

Human infection is acquired in forests from infected ticks which act as both a reservoir of infection and a vector in the maintenance of animal reservoirs of the virus. Infection occurring within populated areas may be transmitted in other ways. Infection by ingestion of goat's milk, as noted above, occurs and familial outbreaks of the disease have been described. It has also been found that oral transmission occurs within pheasant flocks, and this virus apparently has an appreciable potential for infection by ingestion.

LOUPING ILL

Louping ill is an encephalomyelitis of sheep characterized by cerebellar ataxia and necrosis of the Purkinje cells of the cerebellum similar to that observed in Japanese B encephalitis. The term louping (leaping) refers to the peculiar gait of affected animals. The disease occurs in the spring in Scotland and the northern border counties of England. Human infection has been found in Northern Ireland, 89 where it has been confused with poliomyelitis, and in Sweden. 173

The virus is 15 to 22 m μ in diameter and may be grown in chick embryo tissue culture and on the chorioallantoic membrane of the embryonated egg to produce death of the embryo. It produces fatal encephalitis on intracerebral inoculation in mice, and laboratory and field mice (voles) may be infected by peripheral inoculation. Rhesus monkeys, hamsters, and rats may be infected, the last giving a symptomless infection with viremia. Rabbits and guinea pigs are resistant to infection.

Human infections have occurred in laboratory personnel, and in both man and the sheep the disease is diphasic. In the first phase there is viremia, and in man it is accompanied by fever, headache, malaise, gastrointestinal disturbance, and prostration. After clinical improvement there is a recrudescence with fever and signs and symptoms of encephalitis. Recovery has been complete in the human cases which have been observed.

The virus is transmitted by the tick *Ixodes ricinus*. Ticks may be infected by sheep during the viremic stage of the disease, and the infection is congenital in the tick. Whether there are mammalian reservoirs of infection, such as Microtus, is not clear.

Neutralizing and complement-fixing antibodies are produced in response to infection and to immunization with vaccine. There is almost complete cross-protection between this virus and that of RSSE. The two viruses are not identical immunologically and, according to Casals, differences are evident as somewhat higher homologous antibody titers and a significant difference in protection when the challenge inoculum is given peripherally rather than intracerebrally in mice. There would appear also to be differences in the pathogenicity of the two viruses for man.

KYASANUR FOREST DISEASE^{107, 170}

This disease appeared in the Kyasanur Forest in the Shimoga district of Mysore,

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India, in 1956, and was studied for the first time in 1957. Widespread deaths occurred in native monkeys, the langur monkey (*Presbytis entellus*), and the bonnet monkey (*Macaca radiata*), and there was associated disease in man. During the summer (February-April) of 1957 the disease extended over an area of about 500 square miles, and there were 500 human cases of the disease with 70 deaths.

The disease in man does not simulate encephalitis but rather involves the hematopoietic system. Hemorrhage is the obvious major clinical symptom, with evidence of massive hemorrhage in the chest cavity in fatal cases. The source of infection appears to be monkeys,⁷² and possibly rodents are infected also. The infection is not transmitted by mosquitoes, but there is reason to believe that Haemaphysalis ticks may be the vector.¹⁶¹ An accidental infection occurred in the laboratory in the absence of an insect vector.

A virus was isolated from monkeys and also from the blood in human cases of the disease by Work, Trapido, and their colleagues. 169, 172 The viremic period in man is relatively prolonged, and the blood has been found to be infectious from two days before to as long as 10 days after onset of the disease. The KFD virus is cultivable in chick embryo tissue culture and produces cytopathic effects, and the mouse may be infected by intracerebral inoculation to produce a disease in three or four days characterized by prostration, hind limb paralysis, and tremors. Antibody-fixing complement in the presence of mouse brain antigen and neutralizing antibody demonstrable in the mouse are found in convalescents. The virus appears to be antigenically closely related to, but not identical with, the RSSE virus.

A similar hemorrhagic disease, Omsk hemorrhagic fever, has been described in Russia^{86, 94} as of similar etiology. Observations such as these suggest that the virus of RSSE is a member of a group or complex of closely related viruses, some of which produce encephalitis in man with the characteristic paralysis described above, but louping ill virus infections in man are not predominantly encephalitic, and infections with still other of these viruses are characterized by hemorrhagic symptoms.

POWASSAN VIRUS

Powassan virus was isolated from a fatal case of encephalitis in Ontario in 1958 by intracerebral inoculation of suckling mice. The proved to belong to hemagglutinin group B and closely related to RSSE, the first member of the group to be isolated in the Americas. It is found in various forest mammals and their ticks in Canada, and has also been found in New York state. Apparently the same virus was isolated from pools of Dermacentor andersoni collected in Colorado some years earlier, but not identified until the Powassan isolate was described. 154

COLORADO TICK FEVER

Colorado tick fever (American mountain fever, mountain fever, mountain tick fever, tick fever, nonexanthematous tick fever) occurs in the Rocky Mountain regions of the United States where the tick vector is prevalent. The disease in man was described as a clinical entity in 1930 but probably accounted for some part of febrile disease reported by military personnel in that area in earlier years.

The viral nature of the etiological agent was established by serial transmission of the disease in man and in hamsters by the parenteral inoculation of blood, serum, and filtered preparations in 1944. In 1947 it was adapted to the mouse by intracerebral passage in the suckling animal, and the mouseadapted virus grown in the yolk sac of the embyronated egg, where the virus is found largely in the central nervous system of the embyro. Such embryo-adapted strains are of reduced virulence for man. It has also been grown with CPE in cultures of KB epithelial carcinoma cells109 and in L and FL cells after adaptation to the mouse brain. 159 The virus has been isolated directly from infected human or monkey blood or from ticks by this tissue culture method. There is some discrepancy in the reported size of the virus; according to one report is it is extremely small, 10 m μ , and to another 35 to 50 m μ . Its chemical and physical properties have been described by Trent and Scott. 160

It is set apart serologically from the other tickborne viruses of encephalitides in that it is not a member of group B, nor is it related to the other defined HI groups; it is considered to be an ungrouped arbovirus.

On intracerebral inoculation of mice and hamsters, the virus produces lesions similar to those of louping ill virus, *i.e.*, subcortical vascular engorgement and petechial hemorrhages in the cerebrum and destruction of the Purkinje cells.¹⁰⁴ Cytoplasmic inclusion bodies are found in the Purkinje cells and in the neurones of the hippocampus and pons. The experimental infection in the rhesus monkey, by the intravenous or subcutaneous routes, is essentially asymptomatic, but viremia occurs and persists for four to five weeks.⁴⁴

The disease in man. The disease in man is diphasic and is characterized by a sudden onset with chills and aching, and the symptoms include headache, backache, anorexia, nausea, and muscular pains. With the appearance of symptoms there is a febrile reaction which persists for about two days, and a period of remission occurs, with recrudesence in two or three days. Convalescence may be relatively prolonged but is without complications, and recovery is complete; no deaths from the disease have been reported.

Immunity. Complement-fixing¹⁵² and neutralizing⁴³ antibodies are produced in

response to infection, appearing by two weeks after onset of the disease, and persisting for at least as long as three years. Experimental inoculation of human volunteers has shown that there is a solid immunity to reinfection. Active chick embryo-adapted virus used as an immunizing agent produces a solid immunity, and formalin-inactivated virus purified from mouse brain has also been prepared. 155

Epidemiology. The wood tick, Dermacentor andersoni, is naturally infected with the virus, the infection is congenital, and the tick is a reservoir of infection. 120 The virus has been isolated from the dog tick, D. variabilis, on Long Island, but no human cases of the disease have been recognized there, and D. andersoni is considered to be the primary, and possibly sole, vector of the infection. The virus also occurs in a vertebrate reservoir of infection, in the Colombian ground squirrel, Citellus comunbianus, as indicated by serological evidence, and has been isolated directly from the goldenmantled ground squirrel, Citellus lateralis lateralis, and from the porcupine, Erethizon dorsatum epixanthum. 12, 13, 34 Human infection is acquired from infected ticks and tends to occur in persons exposed to tick bites by occupation, as in adult males.

The Bunyamwera Group of Viruses

The Bunyamwera virus has provided a nucleus for the serological grouping of a number of subsequently discovered viruses to give an informal group known as the Bunyamwera group which contains a few more viruses than the several so-called small groups of arboviruses. The Cache Valley and Wyeomyia viruses are considered here, but the group also includes the Chittoor, Germistan, Guaroa, Ilesha, Kairi, and Sororoca viruses.

BUNYAMWERA VIRUS

DEPOCATIONS FOR STANKING

This virus was isolated in 1943, from Aedes mosquitoes caught in an uninhabited part of the Semliki Forest, by the intracerebral inoculation of mice. 136 It produces a lethal infection in hamsters inoculated by

the intracerebral or subcutaneous routes but not in guinea pigs by the intraperitoneal route. Embryonated eggs may be infected by yolk sac or amnionic cavity inoculation, and the embryo is killed. Gross lesions are confined to occasional hemorrhage in the brain, where the virus is present in higher concentrations than in the body, and the virus is neurotropic in the embryo. It is 70 to 120 m μ . The virus is pathogenic for man as shown by the occurrence of disease, once as an encephalitis, in two of four volunteers inoculated, ¹³⁹ and naturally occurring infection has been observed. ⁸⁰

CACHE VALLEY VIRUS

This virus was isolated from a pool of Culiseta inornanta collected in the Cache

Valley in northern Utah by intracerebral inoculation of weanling mice, 65 and can be grown in hamster kidney cell culture to produce cytopathic effects. There is serological evidence of infection of horses in the area, but not in man. The virus has also been found in Brazil, and was isolated from a pool of *Aedes scapularis* Trinidad. 31 In Trinidad there was serological evidence of infection in horses and monkeys (Alouatta) and in one-third or more of human serums examined.

WYEOMYIA VIRUS

This virus was isolated by Roca-Garcia¹¹⁷ in Colombia in 1940 from *Wyeomyia mela-nocephala* and has been isolated from man, a human case of mild febrile illness, in Panama.¹⁴⁰ It is 70 to 120 m μ in diamter and produces encephalitis in the infant mouse following intracerebral inoculation. It is feebly pathogenic for the chick embryo, growing only when inoculated intracerebrally, and will not grow in other parts of the embryonated egg.

Other Mosquito-borne Viruses

A considerable number of mosquitoborne arboviruses are known which are serologically independent or are immunologically related to only one or two other viruses. Those of sandfly fever, Rift Valley fever, and the Turlock virus stand apart, while the California virus is the nucleus of a small group containing the Lumbo, Melao, Tahyna, and Trivittatus viruses. Others are the Simbu and related viruses, the Bakau and related viruses, and the Koongal, Mossuril, Nyando, and mosquito-borne Witwatersrand viruses.

SANDFLY FEVER^{121, 122}

Sandfly fever (pappataci fever, phlebotomus fever, three-day fever) is an acute nonfatal disease of man of viral etiology which is transmitted by a species of sandfly, Phlebotomus papatasii. 128 Its geographical distribution is limited by the occurrence of the vector, and it is found in tropical and subtropical regions during the hot dry season. These areas include various portions of the Mediterranean littoral and the Middle and Far East. The occurrence of the disease in Austrian military personnel in the Adriatic region led to the basic studies of the Austrian military commision in 1909 which substantiated the role of P. papatasii as a vector, which had been indicated by earlier epidemiological studies, and established the viral nature of the etiological agent.

The virus has been found to be 40 to 60 m μ in diameter and occurs as two serotypes, the Sicilian and Naples types. It does not infect the usual laboratory animals, including

primates, and is not cultivable in the chick embryo or in tissue culture. It has been established in the mouse by intracerebral inoculation of the newborn mouse, producing encephalitis with lesions occurring predominantly in the hypothalamus and midbrain, after three blind passages in the case of the Sicilian type and one blind passage of the Naples type. By the tenth passage in infant mice, the Sicilian virus produced a fatal infection in the adult mouse and was fully adapted by the twenty-fifth passage. The adapted virus did not produce disease in rhesus monkeys, rabbits, guinea pigs, or hamsters but did induce an immune response. It had also lost pathogenicity for man but produced an immunity to the nonadapted virus.

The Naples strain was not regularly pathogenic for weaned mice until the thirty-fifth passage, and by the fifty-fifth passage was fully adapted, i.e., virus was found in equal titer in both newborn and weaned mice. In contrast to the Sicilian type, the mouseadapted Naples type produced a mild disease in rhesus monkeys on intracerebral inoculation, but did not infect other experimental animals. Both serological types are cultivable in human or mouse kidney cell culture after mouse passage. A cytopathic effect and plaque formation occurs in the human cell cultures after three to four tissue culture passages, but on first passage in mouse cells.62

The disease in man. The incubation period of sandfly fever is usually three or four days. There may be prodromal malaise and abdominal distress. In the febrile period the viremia is of a lower level than in

dengue, with perhaps 1000 MID per ml. of virus present in the blood. The symptoms, occurring in various combinations, include nausea, headache, backache, stiffness in the neck and back, pains in the joints, sore throat, and anorexia, but there is no rash as in dengue. The febrile period lasts for two to four days, and during convalescence there may be diarrhea and weakness. Relapse occurs during convalescence in some small portion of cases. The disease is rarely if ever fatal.

Immunity. Two or more attacks of the disease may occur during the same season, and this has led to the belief that an effective immunity is not produced by the infection. Controlled experiments in human volunteers, however, have shown that there is a solid immunity to the homologous virus serotype which persists for a considerable time, but that there is substantially no crossimmunity between serotypes. The development of mouse-adapted strains of both known serotypes has made possible both complement-fixation tests, using mouse brain antigen, and mouse protection tests. and the application of both of these has fully substantiated the antigenic independence of the serotypes. Active immunization of man with mouse-adapted virus has been demonstrated experimentally, and immunization against the disease, and also dengue, may assume some practical importance under certain circumstances, as in military operations.

Epidemiology. There appears to be no vertebrate host other than man, and *P. papatasii* is the only known vector of the infection. There is some evidence which has been taken to suggest that congenital transmission of the infection in the vector may occur, but this has not been established. The seasonal occurrence and short life of the vector, together with the self-limited nature of the infection in man, has been thought to imply some other reservoir of infection, but as yet this remains purely hypothetical.

RIFT VALLEY FEVER¹⁰⁵

Rift Valley fever (enzootic hepatitis) is a disease of viral etiology which was first observed in 1930 in domestic animals and man in the Rift Valley in Kenya, South

Africa. Serological evidence, *i.e.*, mouse-protective antibody, indicates that the infection is widespread in Africa, occurring in Uganda, the Sudan, French Equatorial Africa, and the Union of South Africa, as well as in Kenya.

The virus is 23 to 35 m μ in size and is almost uniquely stable, serum remaining infective for as long as two years at refrigerator temperatures. It is cultivable in chick embryo tissue culture and in the chick embryo and is pathogenic for most experimental animals except the guinea pig, but it does not infect chickens, canaries, or pigeons. Mice are especially susceptible, and the experimental infection is fatal within four days. South American monkeys are somewhat more susceptible than African monkeys and respond with a febrile reaction, while the infection in African monkeys is symptomless although there is viremia. Neurotropic strains of the virus have been produced by intracerebral passage in mice. A variant, described as Lunyo virus, has been isolated from mosquitoes and a monkey in Uganda which, unlike RVF virus, produces an encephalitis in mice associated with intranuclear eosinophilic inclusion bodies.¹⁶⁴ This naturally occurring neurotropic strain could be made viscerotropic by intraperitoneal passage in the mouse.

Natural infection occurs in cattle and sheep, but not horses, with a 10 to 20 per cent mortality in adults and abortion in pregnant ewes. Young lambs are very susceptible to infection, and the mortality rate may be as high as 90 per cent. The characteristic lesion seen at autopsy is focal necrosis in the liver, beginning, like that of yellow fever, in the midzone of a lobule. The degeneration is of a hyaline type, and isolated cells are similar to the Councilman bodies seen in yellow fever. In the fulminating disease in lambs the destruction is very extensive and may involve practically all of the parenchymatous cells.

Naturally occurring disease in man is found in persons associated with infected animals, such as shepherds and veterinarians. The disease seems to be especially infectious for laboratory workers, and there have been a considerable number of such infections. The incubation period is five to six days, and the onset of the disease is abrupt. It resembles dengue, with pain in the extremities and joints that may be extreme. The febrile phase lasts only a few

days, convalescence is rapid, and recovery complete, and the disease is rarely fatal. In both man and lower animals a solid immunity results from the infection, and both complement-fixing and neutralizing antibodies are present in the serum.

The disease is transmitted among animals by blood-sucking arthropods which feed during the night. The mosquito Eretmapodites chrysogaster has been found naturally infected in the Semliki forest in Uganda and shown to transmit the disease under experimental conditions. A. caballus and C. theileri appear to be the natural vectors of the infection in South Africa. 142 Epidemiological observations suggest a wild animal reservoir of infection, but such a reservoir has not been found, and it is not known how the infection is maintained in interepidemic periods. The disease seems not to be transmitted among susceptible animals by contact, and the way in which accidental laboratory infections are acquired is unknown.

CALIFORNIA VIRUS¹¹⁴

Three strains of this virus were isolated in 1943 from pools of A. dorsalis and C. tarsalis by intracerebral inoculation of young mice; they produced encephalitis in these animals, cotton rats, and hamsters. Inapparent infection with viremia was produced in ground squirrels, but calves and chickens were not susceptible. The virus grew well in the chick embryo and in other parts of the embryonated egg, but usually without death of the embryo. It was found to be 60 to 125 m μ in diameter and immunologically distinct from the other arthropodborne viruses. It is unrelated to the main HI groups of arboviruses, but is part of a small serologically distinct group which also contains the Lumbo, Melao, Tahyna, and Trivittatus viruses.

This virus was found only in the San Joaquin Valley in California, and within Kern Country, where it continues to persist in endemic form. 48 There was serological evidence of infection in man, horses, cattle, rabbits, and ground squirrels. This virus was the probable cause of fatal encephalitis in an infant, and the possible cause of disease in two other persons, but has not been

isolated from man or other mammals. It is believed that it occurs as a reservoir of infection in lower animals, and the infection is maintained, and transmitted to man, by mosquitoes to give a symptomless or subclinical infection.

TURLOCK VIRUS84, 165

This virus was isolated from C. tarsalis in California by intracerebral inoculation of infant mice. It grows in the embryonated egg, killing the embryo in two to six days, and may be cultivated in chick embryo cell culture. It was found to be relatively large, 120 to 180 m μ in diameter, and, by the complement-fixation reaction, unrelated to other arthropod-borne viruses except the Umbre virus. It is not known to cause disease in man.

BWAMBA FEVER VIRUS

Bwamba fever is a mild febrile disease of man which occurs in African natives in Uganda. The disease is characterized by abrupt onset with fever, headache, backache, mild conjunctival injection, and rash. The disease persists for only a few days, and convalescence is rapid. Nine strains of the causative virus were isolated from the blood¹³⁷ by intracerebral inoculation of mice, producing hyperemia and edema of the brain with degenerative changes in the pyramidal cells of the cortex. The virus is almost completely avirulent for the mouse by peripheral inoculation. A nonfebrile infection is produced in rhesus monkeys. and guinea pigs and rabbits are not susceptible to infection. The virus is cultivable in the embryonated egg by yolk sac inoculation and is found in highest concentration in the brain and body of the embryo. The embryo is not killed, but evidence of gross hemorrhage is found in the brain. The virus is 70 to 120 mu in diameter and is antigenically distinct. It is antigenically related to Pongola virus but not to the other arboviruses. Neutralizing antibody occurs in the native population in proportions ranging from 13 to 81 per cent of serums examined, suggesting that the infection is widespread.

Interrelationships Among Arboviruses

The arthropod-borne viruses have in common a reservoir of infection in lower animals, a transmission cycle in which the infection is carried by biting arthropods, mosquitoes, or ticks, and a general kind of pathogenesis in which there is viremia in the bird or mammalian host and infection of the viscera or central nervous system. depending upon the relative neurotropism of the virus. As noted earlier, human infection is usually incidental to the natural history of the virus although under certain circumstances, as in Aedes-transmitted vellow fever, man may function as a significant host or even a reservoir of infection. There are also other indications that these viruses are more closely related to one another than to other viruses; for example, all the arthropod-borne viruses which have been tested are inactivated by deoxycholate in 1:1000 dilution, while other neurotropic viruses, including poliovirus, mouse encephalitis viruses, and Coxsackie and encephalomyocarditis viruses (see below) are not. 150

Among these viruses there are groups and subgroups of viruses which resemble one another. As indicated earlier, they fall into two general size ranges, those 15 to 30 m μ in diameter and the larger viruses 70 to 120 m μ in diameter, although there are exceptions such as the Sindbis virus which is intermediate in size. The significance of grouping by size is uncertain, for antigenic relationships, for instance, may occur between the larger and smaller viruses.

Immunological relationships are often of basic significance and are also of practical importance in relation to cross-immunity to infection and to serodiagnosis of disease or serological identification of viruses. These viruses, like many other microorganisms, induce the formation of complement-fixing and neutralizing, or protective, antibodies which may be identical but often are not. In addition, many, but not all, of the arthropod-borne viruses agglutinate goose or neonatal chick red blood cells, and, as in the case of influenza and related viruses, the hemagglutinin is antigenic and stimulates the formation of hemagglutinin-inhibiting antibody. The hemagglutinins and complement-fixing antigens are separable by chromatography, and preliminary studies have indicated that virus strains of groups A and B have two hemagglutinins which are antigenically similar, and two complement-fixing antigens are present in strains of group B, but only one, the virus particle, in group A.⁴⁷

Of the three general kinds of serological reactions which are available for the characterization of these viruses, the hemagglutinin-inhibiting antibody is the least specific in that it gives a high proportion of cross-reactions, neutralizing antibody is highly specific, 112 and complement-fixing antibody is intermediate in specificity.

The application of the hemagglutinationinhibition reaction allows the separation of the arthropod-borne viruses into broad groups²⁶ as noted earlier. Groups A and B, the latter being considerably the larger, were distinguished first. With the isolation of related strains, the previously ungrouped Bunyamwera virus provided the basis for a Bunyamwera group.¹⁹ These groupings are shown in the accompanying table with virus strains illustrating the observed separations. A group C was described, 20 which consists of virus strains isolated in Belem (Brazil). Of the remainder, some fall into minor groups made up of two or three related strains, while others are as vet ungrouped. These groupings are given in detail by Casals.¹⁷ It is probable that this grouping can be simplified somewhat by aggregation of closely related viruses as variants of a designated prototype strain.

Subgroups within the hemagglutinin groups may be distinguished by cross-reactions in the complement-fixation reaction in which the titer of heterologous reactions is commonly less than that of the homologous reaction. For example, West Nile, St. Louis encephalitis, Japanese B encephalitis, and Murray Valley encephalitis viruses are related with respect to complementfixing antigens, and yellow fever virus is related to this group and also to dengue and Uganda S viruses. A Japanese B encephalitis antiserum, having a homologous titer of 1:128, may show a titer practically as high against West Nile antigen and one of 1:32 against yellow fever antigen. Or, yellow fever antiserum having a homologous titer of 1:28 may fix complement in titers as high

Hemagglutinin Groups of Arthropod-borne Viruses

GROUP A	GROU	ЛР В	BUNYAMWERA GROUP
EE viruses	St. Louis E	West Nile	Bunyamwera
Mayaro	Yellow fever	Uganda S	Cache Valley
Semliki	Louping ill	Ilheus	Germiston
Uruma	Russian SSE	Powassan	Guaroa
Chikungunya	Kyasanur Forest	Bussuquara	Ilesha
Sindbis	Zika	Dengue viruses	Kairi
O'nyong-nyong	Japanese B	Wesselsbron	Wyeomyia
Middelburg	Murray Valley	Ntava	Chittoor

as 1:32 in the presence of West Nile or Japanese B encephalitis antigen. Similarly, a dengue antiserum having a homologous titer of 1:128 may fix complement with yellow fever antigen in dilutions of 1:8 to 1:32, in the presence of West Nile antigen in dilutions of 1:16 to 1:64, and in the presence of Japanese B encephalitis antigen in dilutions of 1:16 to 1:64. Similar cross-reactions occur between the western and eastern varieties of equine encephalomyelitis virus.

When the more highly specific neutralizing antibodies give heterologous reactions, it is generally taken to be indicative of an extremely close relationship between the viruses. There is, for instance, a partial cross-neutralization between the western and eastern equine encephalitis viruses, and a similar relationship among the West Nile, Japanese B encephalitis, St. Louis encephalitis, and Murray Valley encephalitis vi-

ruses. In the case of Russian spring-summer encephalitis, louping ill, and Kyasanur Forest viruses, the relationship is very close and the differences among these viruses are considered to be of little more than variant status. Semliki Forest virus, Kumba virus, and Mayaro virus give essentially complete cross-neutralization reactions and are therefore regarded as practically identical, and Chikungunya virus is either identical with or closely related to them. These relationships, together with those evident in the complement-fixation reaction, are indicated as subgroups within the hemagglutinin groups in the accompanying table. Such subgroups may not, however, fall entirely within the hemagglutinin groups; the Chikungunya virus, for instance, although closely related to Semliki Forest virus, also cross-reacts to a minor degree with dengue type 1 virus.

Lymphocytic Choriomeningitis

This disease was first observed in 1934, by Armstrong and Lillie, who isolated the virus in a monkey being used in the study of St. Louis encephalitis. The choroid plexuses and meninges were involved, and there was lymphocytic infiltration, these characteristics giving the disease its name. The following year it was found by Traub¹⁵⁸ to be widespread as an endemic, often symptomless, infection in mice, and in 1936 the virus was isolated, both in this country and in England, from human cases of disease diagnosed as aseptic meningitis.

The virus is not an arbovirus in that, so far as is known, it is not arthropod-borne under natural conditions, although Aedes mosquitoes and Cimex can transmit the disease under experimental conditions and it has been found in ticks and the progeny of infected ticks. It resembles the arboviruses in that it is of the same order of size, 40 to 60 m μ and is ether-sensitive and pH labile. It occurs as a single antigenic type. It has been suggested that it may be an arbovirus modified in such a way that it is not usually transmitted by arthropods. It may be grown in mouse or chick embryo tissue culture⁵ and in the embryonated egg on the chorioallantoic membrane or in the yolk sac. In the egg it does not kill the embryo, and the egg may hatch and the chick survive. Strains of high virulence may grow in chick embryo cell monolayers to give plaques on prolonged, 12-day, incubation.

Pathogenicity. 85, 132 The human disease occurs during the cold months of the year, chiefly in adults 20 to 40 years old. It varies in severity from a mild influenza-like disease, through aseptic meningitis or meningoencephalitis, to a fatal systemic form of the disease. The respiratory type of disease may not progress further, and few such cases are recognized as infections with this virus. Or the respiratory symptoms may be prodromal, and the disease progress with the appearance of central nervous system involvement within a few days, including sudden severe headache, fever, nausea, and drowsiness, or the meningitis may occur without prodromata. In the severe form of the disease, which occurs relatively rarely, the symptoms are referable to the portions of the central nervous system affected. During the febrile stage of the disease the virus is present in the blood, nasopharyngeal secretions, and urine, and in the spinal fluid in the meningeal type of disease. Complications are rare and recovery is usually complete.

In the naturally occurring infection in mice two kinds of disease are observed.66 In the one the disease takes a conventional course with the production of complementfixing antibody, and there is persistence of small amounts of virus in various organs. When infection occurs in utero or soon after birth, the resistance of the animals is high and the infection is symptomless. There is apparently no detectable immune response, but the animals are highly resistant to superinfection, and virus is present in large amounts in blood and tissues. The latter kind of infection is considered by some to represent acquired immune tolerance to the agent (see below). In any case, the infection persists in endemic form, and carrier mice represent a reservoir of the virus.

Immunity. Both complement-fixing and neutralizing antibodies are formed in response to the infection. Both appear relatively late in the disease, complement-fixing antibody reaching diagnostic levels in three to four weeks and disappearing within a few months, and neutralizing antibody reaching diagnostic titer in seven to eight weeks after onset of the disease and persisting

for a period of years. The occurrence of immunological tolerance appears to be a significant factor and has been studied at some length. 162

Experimental infections. A number of experimental animals are susceptible to infection, including mice, guinea pigs, dogs, monkeys, and chimpanzees, but rabbits and birds are resistant. The mouse and guinea pig are the experimental animals of choice. The mouse may be infected by intranasal or intracerebral inoculation88 to produce a fatal infection; tremors and convulsions develop in one to two weeks with death two or three days later. Peripheral inoculation, as in the footpad,67 results in a nonfatal infection unless very large doses of virus are given by the intraperitoneal route. The occurrence of virus in the kidneys168 is consistent with the shedding of virus in the urine. Fatal disease is produced in the guinea pig by intracerebral inoculation and may also occur following peripheral inoculation. In both kinds of animals virus is present in the nasal secretions, urine, and feces, and postmortem findings include areas of lymphocytic infiltration in the central nervous system and in the organs, and there may be hepatitis.

Epidemiology. Mice, dogs, and monkeys have been found to be naturally infected with the virus, and it is probable that human infection is most often contracted from mice. The mouse infection is endemic, with infection transmitted *in utero* or shortly after birth and maintained by healthy carriers.¹⁵⁸

It is not clearly established in what way man contracts the infection from the mouse or other animal. It is generally believed that the portal of entry is usually the respiratory tract and that the infection is acquired by inhalation; laboratory infections have occurred which were probably acquired in this way.

The infection in man is doubtless much more common than the number of clinical cases of the disease suggest. Armstrong found that 11 per cent of 2000 persons examined at random had neutralizing serum antibody, and it is probable that many immunizing infections are symptomless or are assumed to be influenza or related respiratory disease.

DURAND'S DISEASE

This is a febrile disease characterized by headache and upper respiratory, meningeal, and gastrointestinal symptoms which Durand³³ described as occurring in himself and in two human patients who were inoculated for pyrotherapeutic purposes. Later Findlay³⁶ acquired the disease as a consequence of a laboratory infection.

During the febrile stage of the disease, lasting for less than a week, the blood is infective for the guinea pig by intracerebral or other routes of inoculation. The experimental disease is characterized by adenopathy, pneumonitis, and enlargement of the spleen, and the fatality rate is about 10 per cent. Mice, hamsters, dogs, cats, and monkeys are also susceptible to experimental infection. In both man and experimental animals complement-fixing and neutralizing antibodies appear in the serum after about a week. The virus is 40 to 60 m μ in diameter, cultivable in chick embryo tissue culture, and on the chorioallantoic membrane of the embryonated egg, to give an infection which is lethal to the embryo. It is immunologically unrelated to the virus of lymphocytic choriomeningitis.

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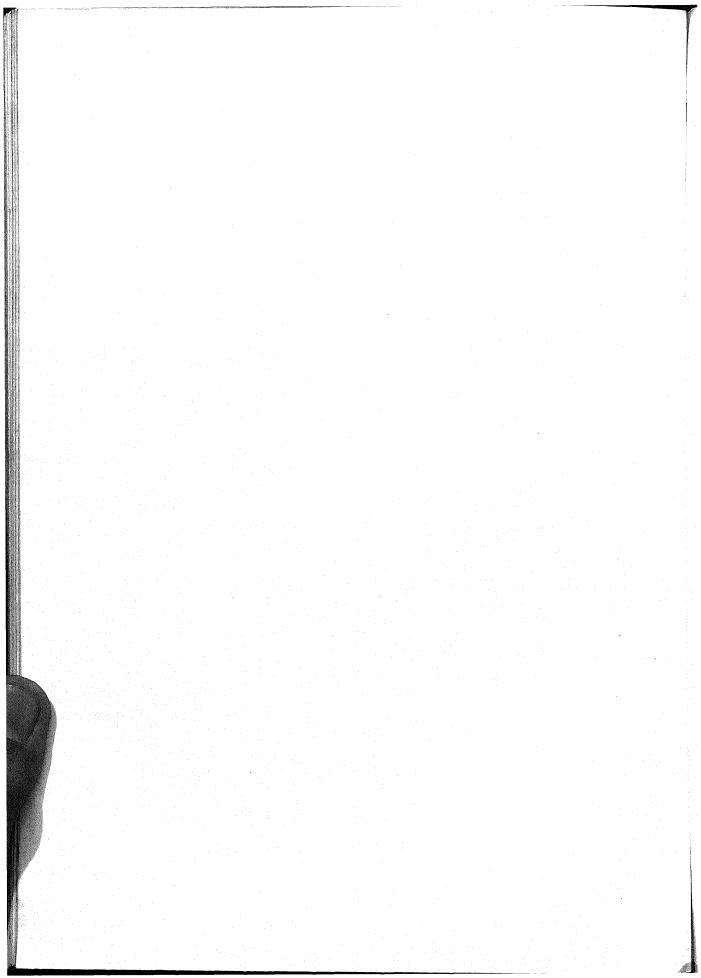
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INDEX



INDEX

Actinomycosis (Continued) A Antigen(s), 346, 486, 549 Ablastin, 781 Abortin, 411, 551 Abortion, in cattle, 550 contagious, 776 in ewes, 846, 852 in goats, 550 in swine, 551 Abortus Bang Ring probe test, 554 ABR test, 554 Abscess, brain, 508 Absorption, agglutination, reciprocal, of oxygen, chemical, 23 Acanthocheilonema perstans, 818, 820 A. streptocerca, 818, 820 Acceptor(s), energy, 114 hydrogen, 106 Acetobacter suboxydans, 171, 201 Acetyl-coenzyme A, reactions of, 127 Achalme, 633 Acid(s), disinfecting, 185. See also under specific names, as Amino acid(s). Acid-fast stain, 20 Acne, 661 Aconitase, 129 Actinobacillosis, 574 Actinobacillus, 570-575 Actinobacillus actinoides, 574 A. actinomycetem-comitans, 574 A. ligniersi, 574 A. mallei, 411, 570-573. See also Glanders bacillus. A. pseudomallei, 573 A. whitmori, 573 Actinomyces, morphology, in culture, 693 pathogenicity, for animals, 694 for man, 694 Actinomyces bovis, 574, 691, 693, 694 A. eriksonii, 691 A. graminis, 691 A. hominis, 691 A. israeli, 689, 691, 693, 694, 696 A. maeslundi, 693 A. muris, 602 A. muris-ratti, 602 A. necrophorus, 601 A. rhusiopathiae, 598 Actinomycetes, morphology of, 690, 691 pathogenic, 690-700 serology of, 694 Actinomycosis, 574, 691-695

Acute laryngotracheobronchitis virus, Acute respiratory disease, 885, 893, 937 parainfluenza viruses and, 893 Adaptation, enzyme, 236-238 induced, 245-247 microbial, 228 biochemical, 229 Addison's disease, tuberculosis and, 669 Adenoid degenerative viruses. See Adenovirus(es). Adeno-pharyngeal-conjunctival viruses, 935 Adenovirus(es), 73, 913, 935-937 antigenic structure of, 936 pathogenicity for man, 937 Adhesion, serological, 371 Adiospiromycosis, 729 Adjuvant(s), antigenic, 336 Freund, 336 Adsorption of viruses, 87 Aedes abnormalis, 948 A. aegypti, 948, 953, 954, 956, 958, 959 A. africanus, 957, 959 A. albopictus, 956 A. caballus, 967 A. dorsalis, 967 A. ingrami, 956 A. longipalis, 956 A, mitchellae, 947 A. natronius, 956 A. polynesiensis, 819, 956 A. scapularis, 965 A. scutellaris, 956 A, simpsoni, 959 A. taenior Lynchus, 956 A. vexans, 956 Aerobacter aerogenes, 137, 315, 485 A. cloacae, 315 Aerobes, obligate, 111, 248 spore-forming, 614-621 Aeromonas hydrophila, 479 A. salmonicida, 479 A. shigelloides, 479 Aerosols, disinfecting, 190 Aflatoxin, 738 African sleeping sickness, 749, 777, 778 diagnosis of, 778 epidemiology and control of, 778 immunity to, 778

immunity to, 695 isolation and diagnosis of, 695

Agalactia, contagious, 608 Agar. See also Culture medium(s). Avery's oleate hemoglobin, 579 blood, 16 brain-heart infusion, for enterotoxin, 420 chocolate, 16 cystine blood, 16 desoxycholate-citrate, 502 eosin-methylene blue, 17 Fildes', 579 Kligler's iron, 502 lead acetate, 15 MacConkey, 502 meat extract broth and, 15 meat infusion broth and, 15 Shigella-Salmonella, 502 triple sugar iron, 502 Age, resistance and, 280 Agglutination, 31 acid, 371 group, 369 H. 370 macroscopic, 32 mechanism of, 371 O, 370 passive, 368 spontaneous, 370 Agglutination absorption, reciprocal, Agglutination reaction, cross-reactions to, 369 Agglutination test, 497 microscopic, 31 slide, 367 Agglutinin(s), 367-372 adsorption of, 369 cold, 370 group, 369 H, 511 MG, 441 O, 511 titration of, protocol for, 32 Agglutinogen, 367 Air, bacteria from, water and, 311 displacement by inert gas, 23 Ajellomyces dermatitidis, 723 Alanine, biosynthesis of, 150 Alastrim, 862 Albert's diphtheria stain, 19 Alcaligenes bookeri, 513 A. faecalis, 513 A. marshallii, 513 A. metalcaligenes, 513

Alcaligenes (Continued)	Ancylostoma braziliense, 814 A. caninum, 814	Anthrax, 6, 614-620. See also Bacillus anthracis.
A. recti, 513 A. viscosus, 513	A. ceylonicum, 814	bacteriological diagnosis of, 619
Alcohol, as disinfectant, 189	A. duodenale, 812, 813, 814	chemotherapy of, 619
butyl, fermentation of, 137	life cycle of, 812	cutaneous, 618
fermentation of, 136	Andaman A fever, 765	immunity to, 619
Aleukia, alimentary toxic, 739	Anderson, 404	immunization against, 620
Alexin, 377	Anderson's medium, 650	intestinal, 619
Algae, blue-green, 717	Andrewes, 934	pathogenicity, for lower animals, 616
Alkalis, disinfecting, 185	Anemia, in animals, Clostridium welchii	for man, 617
Alkylating agents, 190	and, 636	pulmonary, 618
Allantoic cavity, propagation of micro-	infectious, 603	symptomatic, 641
organisms in, 26	tapeworm, 808	toxins of, 616
Allergen, 408	Angina, monocytic, 873	vaccine, 620
Allergy, atopy and, 408-411. See also	Vincent's, 593, 601, 750	Antibacterial activity, antimetabolite
Hypersensitivity.	Angiostrongylus cantonensis, 815	theory of, 201-204
contact, 410	Animals, anemia in, Clostridium welchii	Antibiotics, 204-210. See also Chemo-
drug, 410	and, 636	therapeutic drugs.
food, 410	as vectors of brucellosis, 552	antagonists of, synthesis of, 235
forms of, 409	bartonellosis of, 605	as feed supplements, 215
heredity and, 409	domestic, resistance and, 278	broad-spectrum, 207
nonatopic, 408	tuberculosis in, 676	cell wall synthesis and, 209
to dander, 410	experimental, Actinomyces and, 694	chemical classification of, 208
to pollen, 410	resistance and, 278	combined, therapy with, 213
Allescheria boydii, 701	tuberculosis in, 676	from bacteria, 205
Allodermanyssus sanguineus, 840	germ-free, 289	from higher fungi, 205
ALTB virus, 894	inoculation of, 24–25	in food preservation, 215
Amanita muscaria, 739	lower, bacillary dysentery and, 523	in tuberculosis, 672
A. patherina, 739	blastomycosis and, 724	inhibitors of, decomposition of, 235
A. phalloides, 738, 739	Brucella and, 550	macrolide, 208
A. verna, 739	cholera vibrio and, 541	mechanisms of action, 209
Amblyomma americanum, 838	Clostridium novyi and, 638	microorganisms dependent on, 234
A. cajennense, 838	Clostridium welchii and, 637	permeability of, 209
A. haebreum, 839	Coccidioides immitis and, 727	polyene, 208
Amebae, intestinal, 771–775	coliform bacilli and, 487	protein synthesis and, 209
Amebiasis, 772	dengue and, 954	resistance to, 231–236
chemotherapy of, 773	diseases of transmissible to man,	Antibody(ies), 347–356
diagnosis of, 773	307 EGHO wirmons of 027 028	allergic, 410
Amebic dysentery, 772	ECHO viruses of, 927–928	antibacterial activity of, 389
Ameloid trophozoite, 784	gonococci and, 469	antiserum and, 347
American mountain fever, 963	Hemophilus pertussis and, 584	avidity of, 355
Amino acid(s), bacteria and, 174 assimilation by, 174	hepatitis of, 934–935	formation of, 351
factors in requirements for, 175	herpetic infections of, 870	by single cells, 394
biosynthesis of, 153	Histoplasma capsulatum and, 732 influenza in, 891	cell-mediated, 393 in vitro, 393
regulation of, 156	lymphogranuloma venereum and,	in vivo, 395
breakdown of, 143–145	854	radiation and, 393
deamination of, 144	meningococci and, 475	rate of, 352
decarboxylation of, 143	poxviruses of, 866	sites of, 393–397
metabolism of, 142–157	psittacosis and, 851	spleen and, 394, 396
by obligate anaerobes, 145	rabies in, 905	theories of, 351
synthesis of, 145	Salmonella and, 502	heterogeneity of, 354
vitamins and, 175	staphylococci and, 425	in colostrum, 403
p-Amino acids, racemization and im-	streptococci and, 439	in milk, 403
portance of, 146	tetanus in, 628	incomplete, 355
p-Aminobenzoic acid, bacteria and, 171	Treponema and, 752	maternal, 403
Aminohydroxybenzoic acids, 198	postmortem examination, 25	natural, 398
Ammonia, uptake of, 142, 145	Animal passage, restoration of virulence	nature of, 347
Ammoniacal silver nitrate, preparation	by, 229	protective and neutralizing, 384-385
of, 20	Animal viruses, 72–75	purification of, 356
Ammonium oxalate crystal violet stain,	classification of, 257	secondary response of, 253
19	developmental stages, 94	to enzymes, 375
Amnionic cavity, egg, propagation of	egg culture of, 92	valence of, 357
microorganisms in, 26	recombination in, 239	Antibody-antigen reaction. See Antigen-
Anaerobes, facultative, 111	replication of, 92–99	antibody reaction.
obligate, 111, 248	tissue culture of, 93	Antigen(s), 335-347
cultivation of, 22	Anopheles, control of, malaria and, 789	A, 346, 486, 549
metabolism of amino acid by, 145	Anopheles atroparvus, 789	alien nature of, 338
spore-forming, 623-646. See also Clostridium.	A. bellator, 789	alicigio, 410
Anaerobiosis, control of, 23	A. crucians, 947	armiciai, 343
Analysis, antigenic, 370	A. gambiae, 789, 819	B, 346, 486
Anaphylaxis, 404–408	A. maculipennis, 789	blocking, 372
haptenes in, function of, 406	A. minimus, 789	blood group, 339
local, 407	A. punctipennis, 956 A. quadrimaculatus, 789, 956	heredity and, 340 common, 347, 398
passive, 405	A. sollicitans, 947	complete, 335
cutaneous, 407	Antagonism, synergism and, 214	degradation of, 337
		Objectivit Oi, 557

Antigen(s) (Continued)	Aotus trivirgatus, 928 APC viruses, 73, 935	Bacillus(i), 54, 614-621
dilutions of, preparation of, 30 drugs as, 345	Apodemus speciosus, 765	anaerobic, nonspore- 602
F, 535	A. sylvaticus, 764, 765	Battey, 683
flagellar, 496	Apoenzyme, 107	Boas-Oppler, 593
specific and nonspecific, 497	Appendicitis, gangrenous, 636	Bordet-Gengou. See
Forssman, 341, 368	perforative, 636	tussis.
H, 346, 486, 496, 497, 511, 535	Arboviruses, 74, 942–969	butter, 683
heterogenitic, 341	interrelationships among, 968–969	coliform. See Coliforn
of microorganisms, 347 heterophile, 398	ARD, 885, 893, 937 parainfluenza viruses and, 893	diphtheria. See Dipht. theria bacillus.
Hikojima, 535	Argas persicus, 750	diphtheroid, 660-661
immunizing, essential, 390	Arginine, biosynthesis of, 152	Döderlein's, 592
Inaba, 535	Arthritis, streptococcal, 445	Ducrey's, 586-587
intermediate, 535	Arthus phenomenon, 407	Duval's, 521
isophile, 339	Ascariasis, 810	dysentery, 516-528
J, 535	Ascaris lumbricoides, 810	enteric, 479–528
K, 346, 486 killed bacteria as, 336	bronchopneumonia and, 811 Aschoff bodies, 447	classification of, 48 differentiation, biod
L, 346, 486, 860	Ascoli test, in anthrax, 619	Flexner, 516, 526, 52
LS, 860	Ascomycetes, 701	Friedländer's, 485, 48
M, 436, 440, 441, 442, 549	Aspartic acid, biosynthesis of amino	immunological type
microorganisms as, 345-347	acids derived from, 147	pathogenicity of, 4
differentiation of, 346	Aspergilloma, 736	fusiform, 40
living attenuated, 336	Aspergillosis, 735–737 bronchopneumonic, 736	Hansen's, 663 Hiss-Russell Y, 516
middle, 535 multiple, 402	Aspergillus, 701	Hofmann's, 660
NP, 860	Aspergillus flavus, 736, 738	hog cholera, 504
O, 346, 486, 496, 497, 511, 535, 830	A. fumigatus, 735, 736, 737	Johne's, 411, 663, 682
Ogawa, 535	A. nidulans, 736	Klebs-Löffler, 649
organ-specific, 342	A. niger, 736	Koch-Weeks, 581-58
original, 535	A. parasiticus, 738	Lange-Sachs, 517
partial, 336	Asterococcus canis, 608 A. mycoides, 608	Lavington I, 520 Morax-Axenfeld, 586
phylogeny of, 338 polyvalent, 402	Asteroidin, 696	Morgan's, 491, 492
properties of, 337–338	Asthma, 409	Newcastle-Mancheste
R, 436	Athlete's foot, 708	Nocard's, 850
S, 860, 888, 896	Atopens, 410	Novy's. See Clostridi
size and nature of, 337	Attonuation microbial 228	of malignant edema,
somatic, 496 specificity of, 338–343	Attenuation, microbial, 228 Aura viruses, 943	of mouse typhoid, 50 paracolon. See <i>Parac</i>
altered, 343	Australian X disease, 952	parashiga, 516
chemical basis of, 343-345	Australorbis glabratus, 799	Pfeiffer's, 578-581.
species, 338	Autoclave, sterilization by, 14	philus influenzae.
structure of, 497	Autograft(s), 340	Preisz-Nocard, 661
T, 436	facultative, 248	pseudo-anthrax, 620 pseudodysentery, 519
titration of, protocol for, 34 V, 561, 888, 896	Auto-immune disease, 341 Auto-interference, virus, 100	Rabaul, 519
valence of, 357	Autotroph(s), facultative, 132	Rio, 519
variant, 535	obligate, 132, 248	rotlauf, 598
Vi, 497	Autumn fever, 764	saprophytic acid-fast
virulence, 497	Avery, 9	Schmitz, 516, 518
W, 561	Avery's oleate hemoglobin agar, 579	Schmorl's, 601
Antigen-antibody reaction, 356–362, 364	Avian distemper, 897 Avian influenza viruses, 891	shape of, 40 Shiga, 516
analysis of, mathematical, 360	Avian leucosis, 881	Shiga-Kruse, 517
complex formation of, 358	Avian neoplastic disease, 880-882	slipping of, 41
intermolecular, 357	Avian pest, 897	smegma, 683
shock and, 406	Avian pneumoencephalitis, 897	snapping of, 41
specificity of, 357	Avian psittacosis, 851	Sonne's, 521, 525, 52
Antigenic analysis, 370 Antigenic mosaic, 369		Sonne-Duval, 521 Strong, 516
Antigenicity, relative, 336		tetanus. See Clostridi
Antiglobulin reaction, 368	B antigens, 346, 486	timothy, 683
Antihistamines, shock and, 407	B virus, 867, 870	tubercle. See Tubercl
Antileucocoidin, 425	Babes-Ernst granules, 59	typhoid, 503
Antimetabolite theory of antibacterial	Bacillary dysentery, 522-528	vole, 682
activity, 201–204 Antiserum, antibodies and, 347	bacteriological diagnosis of, 524 carriers of, 522	Bacillin, 205 Bacillus abortus, 547
Antistreptolysin O, 442	chemotherapy of, 525	B. aerogenes capsula
Antitoxin(s), 373–376	epidemiology of, 525	B. agni, 634
diphtheria, therapeutic use, 658	immunity to, 527	B. alvei, 614, 621
titration of, 29, 657	pathogenesis of, 524	B. anthracis, 6, 36, 14
for Clostridium novyi, 638	pathogenicity, for lower animals, 523	621. See also An
neutralizing capacity, titration of, 374 Ramon flocculation test of, 375	for man, 522 vaccines for, 527	morphology and s physiology of, 615
scarlet fever, 446	Bacille Calmette-Guérin, 667, 675	toxins of, 616
standardization of, 374	Bacillemia, 627	variations in, 615

e-forming, 599-Hemophilus perorm bacilli. htheria and Diph-480 iochemical, 483 527 487–489, 610 ypes, 488 488 82 82 36 ster, 517, 519 idium novyi. a, 631 504 acolon bacilli. See also Hemo-9 st, 683 26 dium tetani. cle bacilli. latus, 633 146, 175, 614–620, Anthrax. staining, 614

IV	HADEX	
Bacillus (Continued)	Bacteria (Continued)	Bacterium (Continued)
B. anthracoides, 620	growth of, requirements for, 168	Bact. coli neapolitanum, 486
B. botulinus, 642	hemophilic, 578–587	Bact. dispar, 521
B. brevis, 205	in ice, 313 in milk, pathogenic, 324	Bact. dysenteriae, 517 Bact. enteritidis, 504
B. cereus, 237, 615, 620, 621 B. cereus var. mycoides, 621	isolation of, 326	Bact. faecalis alcaligenes, 513
B. coagulans, 621	sources of, 322	Bact. fluorescens non-liquefaciens,
B. erysipelatis suis, 598	external, 323	513
B. firmus, 621	from infected cattle, 322	Bact. funduliformis, 602
B. fragilis, 601	in water, 311	Bact. lactis aerogenes, 323, 483
B. fusiformis, 601 B. lacunatus, 586	factors in, 313 infection of, 88	Bact. monocytogenes, 594 Bact. murisepticum, 598
B. lentus, 621	inorganic elements and, 175	Bact. paradysenteriae, 519
B. megaterium, 57, 620, 621	involution forms, 42	Bact. parashigae, 518
B. mesentericus, 399	ionizing radiation and, 183	Bact. paratyphosum A, 505
B. mycoides, 602, 620	killed, as antigens, 336	Bact. paratyphosum B, 506
B. necrophorus, 601 B. oedematiens, 637	lysogenic, 90 microbiological assay of, 176	Bact. pestis caviae, 504 Bact. psittacosis, 504
B. oedematis maligni Nr. II, 637	molecular oxygen and, 111-112	Bact. radicicola, 513
B. ovitoxicus, 634	morphology of, 24, 39-64	Bact. schmitzii, 518
B. paludis, 634	colonial, 62-64, 221	Bact. suipestifer, 504
B. para-alvei, 614	variation in, 86	Bact. termo, 490
B. parabotulinus, 643	motility of, 18 niacin and, 169	Bact. tularense, 567
B. paratyphosus A, 505 B. paratyphosus B, 506	nitrifying, 141	Bact. typhimurum, 504 Bacteroidaceae, 600
B. perfringens, 633	nomenclature of, 256	Bacteroides, 599-602
B. pertussis, 582. See also Hemophilus	nucleus of, 57	reproduction in, 82
pertussis.	nutrition of, 168–176	Bacteroides fragilis, 601
B. phlegmonis emphysematosae, 633	pantothenic acid and, 171	B. funduliformis, 601, 606 B. fusiformis, 601
B. pseudotuberculosis rodentium, 566 B. pumilus, 621	phagocytosis and, 383 photo-autotrophic, carbon dioxide	B. melaninogenicus, 602
B. ramosus, 602, 620	fixation by, 134	B. pneumosintes, 602
B. seroficus, 621	physical agents affecting, 177-184	B. ramosus, 602
B. subtilis, 57, 111, 178, 191, 205, 272,	pigmentation of, 63	Bakau virus, 965
311, 324, 397, 614, 615, 621	propionic acid, 594	Balantidium coli, 775
B. tropicus, 621 Bacitracin, 205	protoplasts of, 53 purines and, 175	Balkan grippe, 842 Bang, 547, 880
Bacteria, aberrant forms, 42, 224. See	pyrimidines and, 175	Bang's disease, 547
also microorganisms.	radiation and, 182	Bartonella, 64, 603-605
adaptation of, biochemical, 229	respiratory enzymes in, 107-111	systemic position of, 605
amino acids and, 174	revival of, 192	Bartonella bacilliformis, 603
p-aminobenzoic acid and, 171 animal passage of, virulence and, 229	riboflavin and, 170 rickettsia and, 825	immunity to, 605 morphology and staining, 603
as antibiotics, 205	RNA in, 57	pathogenicity, for animals, 605
autotrophic, 132	salts and, 185	for man, 604
biochemical reactions of, 24	sexuality of, 238	physiology of, 604
biotin and, 173	species of, 256	Bartonellosis, epidemiology of, 605
blood coagulation and, 272 cells of, 39	spiral, 41 spore of, 54	of animals, 605 Basidiobolus, 701
internal structures, 54-61	staining of, 18	Basidiobolus meristosporus, 720
staining reactions, 47-49	systematic study of, 23-24	B. ranarum, 720
structure of, 43–62	temperature and, 178	Basidiomycetes, 701
external, 43-47	thiamin and, 168 types of, 256	Bassi, 687
chemo-autotrophic, carbon dioxide fixation by, 134	ultraviolet radiation and, 182	Bastianelli, 783 Bat(s), rabies in, 906
cold and, 181	virulence of, loss of, 229	Battey bacillus, 683
coliform, fermentations of, 137	modification of, 229	Bayne-Jones, 574
cultures of, 21–25	vitamin B complex and, 168	Bazillenemulsion, 673
cytoplasm of, 59 denitrifying, 142	vitamin B ₆ and, 170	BCG, 667, 675
disintegration of, 184	vitamin B ₁₂ and, 173 Bacterial endocarditis, subacute, 440,	Beauvaria, 717 Beauvaria bassiana, 687
dissociation of, 220	449–450	Bebaru viruses, 943
DNA in, 57	Bacterial growth curve, 84	von Behring, 8, 373, 624
drying and, 181	Bacterial metabolism, 105-176	Beijerinck, 5, 7
electrophoresis and, 184 endotoxin-forming, 268	Bacterial respiration, 106–114	Bejel, 757
energy sources for, 107	Bacteriocins, 205 Bacteriophage(s), 69–72_	Beladung reaction, 371 Bergey classification of microorganisms,
enteric, deamination of amino acids	replication of, 87–92	254–255
by, 144	typing of, 417	von Bergmann, 5
exotoxin-forming, 266	Bacterium acidi propionici, 594	Berry-Dedrick phenomenon, 242
folic acid and, 171 freeze-drying and, 182	Bact, actinomycetem comitans, 693	Berzelius, 4
genera of, 256	Bact. aertrycke, 504 Bact. cholera suis, 504	Bignami, 783 Bilharz, 798
gram-negative vs. gram-positive, 48	Bact. coli anaerogenes, 483	Bilharziasis, 798
growth of, 81-86	Bact. coli commune, 483	Binomial expansion, Soper, 301
exponential phase, 85	Bact. coli communior, 483	Biochemical tests, mediums for, 15
lag phase, 84 logarithmic phase, 85	Bact, coli communis, 483	Biotin, bacteria and, 173
autominio hundo, on	Bact. coli mutabile, 230, 483	Birds. See Avian.

Catalase, 111

Bismuth sulfite medium, 17 Borrelia (Continued) Budd, 495 B. novyi, 747 Bumps, 726 Bithionol, 188 Black Death, 558, 559. See also Plague. Bunyanwera virus, 964, 965 B. parkeri, 749 Black disease of sheep, 638 B. recurrentis, 746, 747 Bunsen burner, flame of, sterilization Black fever. See Kala-azar. B. theileri, 748 with, 13 Buruli ulceration, 683 Buschke, 733 B. turicatae, 747, 749 Black leg, 641, 739 of cattle, 633 B. venezuelensis, 747 Blackwater fever, 787 B. vincentii, 750 Busky stunt virus, 78 Botryomycosis, 698 Busse, 733 Bladder worms, 803 Botulism, 329, 642 Blastomyces brasiliensis, 729 Bussuquara virus, 949 B. dermatitidis, 689, 721, 723, 725, cattle, 643 Butter bacillus, 683 immunity to, 646 Bwamba fever virus, 967 morphology of, 723 immunization against, 646 Blastomycosis, European, 733 Bouchet-Gsell disease, 764 keloid, 729 North American, 722-725 Boutonneuse fever, 839 Brain, abscess of, 508 C viruses, 921 Cache Valley virus, 964 black degeneration of, 738 diagnosis of, 725 Brauell, 6, 614 Caignard-Latour, 4 immunity to, 724 Breakbone fever. See Dengue. Calabar, 820 pathogenicity, for lower animals, California virus, 967 724 Breed microscopic count for milk, 325 for man, 723 Brill's disease, 830, 835 Camp fever, 835 South American, 729 British Ministry of Health, drinking Canary pox, 866 water standards of, 317 Candida, pathogenicity of, 714 systemic, 733 Bromine, water purification with, 319 Candida albicans, 213, 687, 696, 714, Blennorrhea, inclusion, 855, 856 Blepharo-conjunctivitis, 586 Bronchocandidiasis, 716 716 Blood, coagulation of, bacteria and, 272 Bronchopneumonia, Ascaris lumbri-C. granuloma, 716 C. krusei, 714 drawing of from animals, 24 coides and, 811 Blood agar, preparation of, 16 epidemic hiberno-vernal, 842 C. paropsilosis, 714 Broth, meat extract, agar and, 15 C. steallatoidea, 714 Blood culture medium, 16 Blood flukes, human, 798 C. tropicalis, 714 meat infusion, agar and, 15 Candidiasis, 714–716 Candidid, 711 Blood groups, antigens and, 339 nitrate, 15 Blue comb, 599
Boas-Oppler bacillus, 593 selenite F, 17 Capsid, structure of, 69 sugar, 15 Capsule(s), 45 Body(ies), Aschoff, 446 tetrathionate enrichment, 17 Bollinger, 860, 866 Borrel, 860, 866 formation of, 222 tryptophan, 15 Bruce, 547, 777 staining of, 20, 46 chromatoid, 771 Brucella, 536, 547-555 virulence and, 275 Capsular reaction, specific, 47 antigenic structure of, 549 defenses of, external, 285 internal, 286 carbon dioxide and, 548 Capsular substance, composition of, 46 Carate, 759-760 Donovan, 610 dyes and, 548 Carbohydrates, complex, 115-117 elementary, 67 hydrogen sulfide and, 548 Guarnieri, 67, 860, 866 metabolism of, 114-138 morphology and staining, 547 inclusion, 67 initial, 67 Carbolfuchsin stain, Ziehl's, 19 pathogenicity, for lower animals, 550 Carbon dioxide, Brucella and, 548 for man, 551 physiology of, 547 toxin of, 549 fixation of, 131-135 L, 223 L. C. L., 67, 850 Negri, 67, 904, 907 autotrophic, 132 by chemo-autotrophic bacteria, 134 variation in, 549 Brucella abortus, 180, 230, 323, 411, 547, 548, 550, 551, 552, 553, 554, normal flora of, 288 by photo-autotrophic bacteria, 134 heterotrophic, 134 Paschen, 67, 860 Boiling, sterilization by, 13 555, 570 importance, 135 Br. bronchiseptica, 555, 578, 583 Br. melitensis, 323, 411, 547, 548, 550, 551, 553, 554, 555, 570 Br. suis, 547, 548, 550, 551, 553, 554 Carbon dioxide tension, 22 water purification by, 319 Carboxydismutase, 133 Boletus satanas, 739 Bollinger bodies, 860, 866 Carboxylation, of propionic acid to Bombyx mori, 75 succinic acid, 135 Boophilus microplus, 842 Br. tularensis, 567 of pyruvic acid to oxalacetic acid, 134 Bordet, 9, 368, 377, 379 Bordet-Gengou bacillus. Carcinoma, mammary, mouse, 880 Brucellergen, 411, 551, 554 He-Brucellin, 411 schistosomiasis and, 800 mophilus pertussis. Brucellosis, 551 Cardiolipin, 755 animal vectors of, 553 bacteriological diagnosis of, 554 Bordet-Gengou phenomenon, 380 Carditis, rheumatic, 447 Bordetella, 578 streptococcal, 445 Bordetella pertussis, 582-586. See also chemotherapy of, 554 Caries, dental, lactobacilli and, 593 Carrier(s), disease, 294 intestinal amebae and, 771 Hemophilus pertussis. cholera and, 398 Bornholm disease, 923 complications of, 552 Borrel bodies, 860, 866 epidemiology of, 552 of bacillary dysentery, 522 hypersensitivity in, 411 Borrelia, 745 of cholera, 537 classification of, 746 of diphtheria, 659 immunity to, 554 in cattle, 550 cultivation of, 746 of Hemophilus influenzae, 580 morphology of, 745 in goats, 550 of meningococci, 473 Borrelia aegyptica, 747 in horses, 551 of pneumococci, 458 of staphylococci, 421, 423 B. anserinum, 748 in swine, 551 B. berbera, 747 insect vectors of, 553 of typhoid fever, 508 B. carteri, 747, 748 vaccines for, 555 cholecystectomy in, 509 B. duttonii, 747 Brugia malayi, 818, 819, 820 types, 98 B. gallinarum, 748 B. pahangi, 820 Carrión's disease, 603 B. hispanica, 747 Brussin reaction, 371 Castellani, 777 B. kochii, 747 B. muris, 766 Bubo, climatic, 853 Cat-bite fever, 872 Cat-bite fever, 872 Cat-scratch fever, 872 tropical, 853

Buchner, 377

B. neotropicalis, 747

Catarrh, contagious nasal, of rabbits,	Chemotherapeutic drugs (Continued)	Cladosporium (Continued)
557	resistance to, in vivo, 231	C. trichoides, 738
Catenabacterium, 600	secondary infections and, 211	C. werneckii, 704
Cattle, abortion in, 550	sensitivity tests, 211	Cladothrix asteroides, 696
contagious, 776	synthetic, 198–201	Clauberg medium, 650
actinomycosis in, 695	Chemotherapy, 9-10. See also under	Claviceps purpurea, 738, 739
anthrax in, 617	specific disease treatment.	Climate, resistance and, 283
blackleg of, 633	Chickens limberness in 330	Climatic bubo, 853
botulism of, 643	Chickens, limberneck in, 330	Clitocybe illudens, 739 Cloaca cloacae, 315, 485
brucellosis in, 550	NDV in, 897 Chickenpox, 871–872	Clonorchiasis, 798
enteritis pneumonia of, 931 infected, bacteria from, 322	Chiclero ulcer, 783	Clonorchis sinensis, 798
Johne's disease of, 411	Chikungunya virus, 948	Clostridium, 54, 623–646
shipping fever of, 558	Chilomastix mesnili, 775	Clostridium acetobutylicum, 137, 201
staphylococcal mastitis in, 425	Chimera, blood type, 342	Cl. botulinum, 145, 184, 312, 329,
CCA virus, 895	Chinese liver fluke, 798	333, 623, 642–646
Celebes vibrios, 532	Chittoor virus, 964	morphology of, 642
Celery blight, 717, 738	Chloramphenicol, 207, 209	pathogenicity for lower animals,
Cell(s), antibody-forming, 393	Chlorine, water purification with, 318	645
bacterial, 39-43	Chloromycetin, 207	for man, 644
internal structures, 54-61	Chlortetracycline, 208	physiology of, 642
morphology of, 221	in food preservation, 215	toxins of, 643
staining reactions of, 47–49	Chocolate agar, preparation of, 16	types of, 643
structure of, 43–62	Chocolate-tellurite medium, 18	Cl. butylicum, 137 Cl. chauvoei, 623, 632, 633, 638,
external, 43–47	Cholecystectomy, in typhoid fever	641-642
body, defense of, general, 392	carriers, 509 Cholera, 504, 536	immunization against, 642
local, 392 inflammation and, 392	Cholera, 504, 536 bacteriological diagnosis of, 538	Cl. edematis, 312
role in immunity, 390, 392	brucellosis and, 398	Cl. feseri, 641
conjugation between, 82	carriers of, 537	Cl. histolyticum, 142, 623, 624, 633,
division of, 81	El Tor, 540	639–640
cessation of, 85	endemic foci of, 539	toxins of, 639
inhibition of, 81	epidemiology of, 538, 539	Cl. kluyveri, 107, 137
synchronous, 85	fowl, 557	Cl. novyi, 623, 631, 637–639, 641
flame, 794	hog, 504	antigenic structure of, 638
HeLa, cultures of, 28	immunity to, 541	antitoxin for, 638
host, tissues and, 98	symptoms of, 537	morphology of, 637
lepra, 678	treatment of, 537	pathogenicity, for animals, 638
response to microorganisms, 288	vaccines for, 541	for man, 638
single, antibody formation by, 394	Cholera nostra, 504	physiology of, 637 toxin of, 638
structure of, variation in, 222 systems of, 391–392	Cholera vibrio, 530-543 antigenic structure, 535	Cl. oedematiens, 637
Warthin-Finkleday, 900	biotypes of, 531	Cl. parabotulinum, 644
Cell wall, osmotic regulation and, 52	classification of, 536	Cl. parabotulinum equi, 643
plasma membrane and, 49-54	Heiberg fermentation types of, 531	Cl. pasteurianum, 142
preparation of, 50	hemolysin of, 535	Cl. perfringens. See Clostridium
properties of, 50	morphology and staining, 530	welchii.
synthesis, antibiotics and, 209	mucinase of, 535	Cl. septicum, 623, 631–633, 638, 641
Celli, 470	pathogenicity, for lower animals, 541	antigenic structure of, 632
Cellular immunity, 390–397	for man, 536	morphology of, 632
Cellulitis, 444	physiology of, 530	pathogenicity of, 633
Cellulose, hydrolysis of, 115	toxins of, 533	physiology of, 632
Cenunda virus, 960 Cephalins, 138	variations in, 536	toxin of, 632
Ceratophyllus fasciatus, 562, 563	Chorioallantoic membrane, propaga- tion of microorganisms in, 27	Cl. sporogenes, 142, 145, 623, 631, 640-641
Cercariae, 794	Choriomeningitis, lymphocytic, 969-	Cl. tetani, 312, 623, 624–630. See also
Cercospora apii, 717, 738	971	Tetanus.
Cercosporamycosis, 738	epidemiology of, 970	antigenic structure, 626
Cerebrospinal fever, 470	immunity to, 970	morphology of, 624
Cestoda, 802-808	pathogenicity for man, 970	physiology of, 625
Chagas, 779	Chromatoid bodies, 771	Cl. welchii, 137, 139, 213, 271, 272,
Chagas' disease, 779, 780	Chromobacter iodinum, 205	313, 314, 315, 331, 332, 389, 397,
diagnosis of, 780	C. violaceum, 205	623, 624, 631, 633–637, 638, 641,
epidemiology and control, 780	Chromoblastomycosis, 717–718	643, 886
immunity to, 780 Chain initiation, 167	Ciliophora, 775	morphology of, 633
Chain termination, 167	Cillobacterium, 600 Circling disease, 597	pathogenicity, for lower animals,
Chancre, hunterian, 753	Circumoval precipitin reaction, 801	for man, 636
soft, 586	Cistron, 238	physiology of, 633
Chancroid, 586	Citellus comunbianus, 964	toxins of, 634
hypersensitivity in, 411	C. lateralis lateralis, 964	types of, 634
Chantemesse, 517	C. pygmaeus, 563	Clouds, infectious, 296
Chemoheterotrophs, 132	C. tridecemlineatus, 605	Coagulase, 272, 420
Chemotherapeutic drugs, 197-215. See	Citrate synthetase, 129	Coastal fever, 621, 765
also Antibiotics.	Citrobacter freundii, 485	Coccidioides immitis, 412, 689, 722,
application of, 210–215 assay of activity, 210	Cladosporium carrionii 717	725, 726, 727, 728
resistance to, 231–236	Cladosporium carrionii, 717 C. mansonii, 704	immunity to, 727
	C. manoning road of the contract contract	parasitic stage, 725

Coccidioides (Continued)	Corynebac
parasitic stage, pathogenicity for	C. equi,
lower animals, 727	C. hofm
for man, 726	C. infant
saprophytic stage, 726	C. minut
Coccidioidin, 412, 727	C. muris
Coccidioidomycosis, 725–728	C. necro
diagnosis of, 728 epidemiology of, 728	C. parvu
primary, 726	C. pseud
secondary or progressive, 727	C. pseud
Coccus(i), pathogenic, gram-negative,	C. pyoge
463-477	C. renale
shape of, 39	C. tenuis
Coctoproteins, 343	C. xeros
Coe virus, 923	Coryzaviru
Coentrus, 806	Contonpox
Coenzyme(s), 107 of pyruvic acid metabolism, 125	Cowdria ru Cowpox, 8
Coenzyme A, 126	Coxiella bi
Cohn, 2	843, 844
Cold, bacteria and, 181	Coxsackie
Coliform bacilli, 483–487	pathoger
differentiation of, physiological, 484-	types of,
485	Crimean h
immunological relationships of, 485-	Cryptococo
487	pathoge: Cryptococ
morphology and staining, 483 pathogenicity, for lower animals, 487	C. laure
for man, 486	C. luteol
physiology of, 484	C. muco
toxins of, 484	C. neofo
variation in, 484	C. neofo
Colitis, ulcerative, 522	Cryptozoit
Collagen disease, 341	Cuban itch
Colonies, bacterial, morphology of, 221	Culex anni
dwarf (D), 221	C. anten C. discal
mucoid (M), 221 rough (R), 221	C. fatiga
smooth (S), 221	C. moles
Colorado tick fever, 963-964	C. pipie
immunity to, 964	C. pipie
Colostrum, antibody in, 403	C. quinc
Columbia SK virus, 928	C. salina
Common cold viruses, 929–930	C. tarsal
Complement, 377 nature of, 378	C. theile
properties of, 377	C. tritae C. univi
titration of, protocol for, 34	Culiseta in
Complement deviation phenomenon,	C. melai
379	Culture(s),
Complement fixation reaction, 33, 380	cell, gro
protocol for, 35	continue
quantitation of, 381	egg, of
Constock, 574	HeLa ce
Conglutinating complement adsorption test, 368	of bacte of fungi
Conglutination, 368	plate, te
Conjugation, cell, 82	pure, 21
recombination and, 238-240	bacte
Conjunctiva, resistance and, 285	24
Conjunctivitis, 582, 937	tissue, 2
inclusion, 846, 856	monk
swimming pool, 856	of an
Coombs test, 368	Culture m
Cooper's pneumococcal types, 456 Copenhagen 222 virus, 894	basal, 1 bismuth
Copepods, 807, 821	chocola
Coproantibody, 396, 397	differen
Coracidium, 807	select
Corper's medium, 665	Dorset's
Corrin moiety, 173	Endo's,
Corynebacteriaceae, 594	enriched
Corynebacterium, 649–661.	in de
Corynebacterium acnes, 661	for biod
C. diphtheriae. See Diphtheria and	for dem
Diphtheria bacillus. C. enzymicum, 661	tion, for stap
	101 stap

INDEX
a
Corynebacterium (Continued)
C. equi, 661 C. hofmannii, 660
C. infantisepticum, 595
C. minutissimum, 697
C. murisepticum, 661
C. necrophorum, 601 C. ovis, 661
C. parvulum, 595
C. pseudodiphtheriticum, 660
C. pseudotuberculosis, 661
C. pyogenes, 661
C. renale, 661 C. tenuis, 697
C. xerose, 661
Coryzaviruses, 913, 929
Cottonpox, 862
Cowdria ruminatum, 834 Cowpox, 864, 866
Coxiella burnetii, 37, 65, 834, 841, 842,
843, 844
Coxsackie viruses, 913, 921-924
pathogenicity for man, 923
types of, 922 Crimean hemorrhagic fever virus, 960
Cryptococcosis, 733–735
pathogenicity for lower animals, 734
Cryptococcus hominis, 735
C. laurentii, 735
C. luteolus, 735
C. mucorugosus, 735 C. neoformans, 689, 721, 733, 735
C. neoformans var. innocuus, 735
Cryptozoites, 784
Cuban itch, 862
Culex annulirostris, 953
C. antennatus, 953 C. discalis, 569
C. fatigans, 819
C. molestus, 951
C. pipiens, 947, 951, 953, 956
C. pipiens var. pallens, 952 C. quinquefasciatus, 819, 951
C. salinarius, 947
C. tarsalis, 945, 946, 951, 967
C. theileri, 967
C. tritaeniorhynchus, 952
C. univittatus, 948, 953 Culiseta inornanta, 964
C. melanura, 947
Culture(s), carrier, 98
cell, growth of tubercle bacilli in, 665
continuous flow, 85
egg, of animal viruses, 92 HeLa cell, 28
of bacteria, 21-25
of fungi, 702
plate, technique for, 21
pure, 21 bacteria in, systematic study of, 23—
24
tissue, 27–29
monkey kidney, 28
of animal viruses, 93
Culture medium(s). See also Agar. basal, 15
bismuth sulfite, 17
chocolate-tellurite, 18
differential, 64
selective and, 16 Dorset's, 16
Endo's, 17
enriched, 16
in deep tubes, 22
for biochemical tests, 15
for demonstration of microbial variation, 227
for staphylococci, 416

Culture medium(s) (Continued) for tissues, 27 Löffler's, 16 Petragnani's, 18 preparation of, 14-18 sterilization of, 13 Wilson-Blair bismuth-sulfite, 502 Cunningham, 771 Cycle(s), life, microbial, 225 replication, of virus, 226 Cyst(s), alveolar, 805 hydatid, 805 osseous, in echinococcosis, 805 Cysteine, biosynthesis of, 150 Cysticercus, 803 Cysticercoid, 806 Cytomegalic inclusion disease, 873 Cytomegaloviruses, 867 Cytopathogenesis, 93 Cytoplasm, of bacteria, 59 Cytotropism, 98 Cystine blood agar, preparation of, 16 Cystitis, 507 Czapek-Dox medium, 702

D colony, 221 Dander, allergy to, 409, 410 Dangerous bug disease, 840 Danish pneumococcal types, 456 Danysz phenomenon, 374 Danysz virus, 504 Davaine, 6, 614 Deamination, 143 nonoxidative, 144 oxidative, 144 Debaromyces neoformans, 735 Decarboxylation, 143 nonoxidative, aldehyde transfer and, 126 oxidative, 127 Dehydrogenases, 10 cytochrome-linked, 108 Delhi belly, 525 Demarquay, 818 Dengue, 948, 953-956 epidemiology of, 956 immunity to, 955 pathogenicity for lower animals, 954 Dental caries, lactobacilli and, 593 Deoxycholate-citrate medium, 17 Deoxyribonucleases, 159 Deoxyribonucleic acid. See DNA. Dermacentor andersoni, 569, 838, 963, 964 D. nuttallii, 839 D. occidentalis, 569, 838 D. parumapterus, 838 D. sylvarum, 839, 962 D. variabilis, 569, 838, 964 Dermatitis, 409 contact, 409 contagious, 697 schistosome, 802 Dermatocandidiasis, 716 Dermatomycosis. See Dermatophytosis-Dermatophilus congolense, 697 Dermatophytes, anthropophilic, 709 differential characteristics, 707 immunity to, 711 pathogenicity of, 708 Dermatophytid, 711 Dermatophytosis(es), 411, 704-712 intertriginous, 708 laboratory diagnosis of, 712 Derriengue, 907

viii	INDEX
Desensitization, 406	Disease(s), age and, 280. Se
Desert rheumatism, 726	specific name, as Bang
Desmodus rotundus murinus, 906	auto-immune, 341
Detergents, as disinfectants, 188	carrier of. See Carrier(s)
Deuteromycetes, 701	climate and, 283
Devil's grip, 923	collagen, 341
Dialister, 600	cytomegalic inclusion, 8'
Dialister pneumosintes, 602	distribution of, age, 306
Diaminopimelic acid, 147	geographical, 306
Diamond skin disease, 598	seasonal, 306
Diamonds, 598	endemic, 293
Diaphorases, 110	epidemic, 293
Diarrhea, acclimatization, 527	etiology of, multiple, 26
infant, 522	fatigue and, 284
Proteus and, 492	foodborne, control of, 3
summer, 931	infectious, 5-7
viral, 931	control of, 307–308 epidemiology of, 293–
Dibothriocephalus latus, 806	incidence of, analysis,
Dichlorophene, 188 Dichuchwa, 757	microbial etiology of,
Dick test, 410, 446	types of, 295–297
Dick test, 410, 446 Dick toxin, 433	insectborne, 307
Dicrocoelium dendriticum, 797	man to man transmissio
Dientamoeba fragilis, 774, 775	neoplastic, 879-880
Dieudonné medium, 538	avian, 880–882
Dihydrostreptomycin, 207	nutrition and, 283
Dihydroxyethyl-DPT, 122	of lower animals tran
Dioxygenases, 110	man, 307
Diphosphothiamin, 125	of milk, 324
Diphtheria, 649-661. See also Coryne-	pandemic, 293
bacterium and Diphtheria bacillus.	pathogenic microorganis
antitoxin for, therapeutic use, 658	289
titration of, 29, 657	periodontal, 593
bacteriological diagnosis of, 656	prevalence of, 306
carriers of, 659	race and, 279
control of, 659	season and, 283
epidemiology of, 659	sex and, 282
immunity to, 656	shellfish and, 332
immunization against, 657	streptococcal, epidemio
susceptibility to, 659	waterborne, control of,
toxin-antitoxin for, 658	Disinfectants, 184–197
toxoid for, 658	actions of, antibacterial
virulence test for, 656	192
Diphtheria bacillus, 649-661. See also	dynamics of, 193
Corynebacterium.	factors influencing, 19
antigenic structure of, 651	mechanisms of, 191
lysogenicity of, 652	gaseous, 190
morphology and staining, 649	inorganic, 185
pathogenicity, for lower animals,	organic, 187
655	specificity of, 193
for man, 654	standardization of, 195
physiology of, 650	Dismutation, 137
toxicity of, 652	Dissociation, bacterial, 22
assay of, 366, 655 toxin of, 651	induction of, 222
types of, 653	S-R, 500
variations in, 653	Distemper, avian, 897
Diphtheria stain, Albert's, 19	canine, 899 DNA, 158
Diphyllobothrium latum, 803, 806	in bacteria, 57
epidemiology and control of, 808	structure and self-duplic
life cycle of, 806	synthesis of, 164
D. mansoni, 808	Döderlein's bacillus, 592
Diplococci, gram-negative, 476-477	Dog(s), anaphylactic shoc
anaerobic, 477	rabies in, 905
pigmented, 477	Domagk, 197
Diplococcus constellatus, 456	Donor(s), energy, 114
D. gonorrhoeae, 463	hydrogen, 106
D. magnus, 456	Donovan, 610, 781
D. morbillorum, 456	Donovan bodies, 610
D. mucosus, 456	Donovania granulomatis,
D. paleopneumoniae, 456	Dorset's egg medium, 16
D. plagarumbelli, 456	Dose-response curve, 265
D. pneumoniae, 453, 455	Dracunculus medinensis,
Dipodomys merriani, 727	Drugs, as antigens, 345
Dipylidium caninum, 806	chemotherapeutic. See
Dirofilaria immitis, 820	peutic drugs.
Disaccharides, 115	hallucinogenic, 738
hydrolysis of, 116	idiosyncrasy to, 410

ge and, 280. See also under Drug addicts, tetanus in, 628 Drug resistance, gonococcal, 466 name, as Bang's disease. staphylococcal, 421 See Carrier(s). streptococcal, 433 Drying, bacteria and, 181 Dubini, 812 ic inclusion, 873 Duck egg virus, 904 Ducrey's bacillus, 411, 586-587 n of, age, 306 Dujardin, 2 Dulbecco and Vogt's PBS, 27 Duran-Reynals factor, 274 Durand, 853 Durand's disease, 971 , multiple, 262 von Dusch, 3 Dust, infectious, 296 inhalation of, tuberculosis and, 670 control of, 333 Dutton, 777 ology of, 293-308 Duval's bacillus, 521 Dyes, as disinfectants, 191 e of, analysis, 301 al etiology of, 259-263 Brucella and, 548 Dysentery, amebic, 522, 772 bacillary. See Bacillary dysentery. an transmission, 307 lamb, 634 swine, 543 Dysentery bacilli, 516-528 classification of, 516 animals transmissible to Large-Sachs, 518 EA 102 virus, 895 c microorganisms and, 259-Eagle's basal medium, 27 Eagle test, 755 Earle's BSS, 27 Eastern equine encephalitis. See EEE. Eaton agent, 609 Eberth, 495 ECBO viruses, 924 ECDO viruses, 924 cal, epidemiology of, 441 e, control of, 310 Echinococcosis, 805 Echinococcus alveolaris, 805 antibacterial, reversal of, E. granulosus, 804 E. multilocularis, 805 ECHO-rhino-coryza viruses, 929 ECHO viruses, 913, 924-926 influencing, 193 antigenic types of, 926 culture of, 924 of lower animals, 927-928 pathogenicity of, 926 ECMO viruses, 924, 927 von Economo's disease, 943 ECSO viruses, 924 Ectromelia, 304, 866 , bacterial, 220 Eczema, epidermic, 697 facial, of sheep, 739 noninfectious, 409 Eczema herpeticum, 868 ED₅₀ dose, 264 Eddy's pneumococcal types, 456 and self-duplication of, 164 Edema, angioneurotic, 409 malignant, 637 bacillus of, 631 phylactic shock in, 405 EEE, 946-947 epidemiology of, 946 vectors of, 947 Egg, embryonated, 25-27 Egg culture, of animal viruses, 92 Egg medium, Dorset's, 16 Ehrlich, 6, 9, 197, 207, 356, 374, 377, 379 granulomatis, 610-611, 853 Ehrlichia bovis, 834 medium, 16 E. canis, 834 ise curve, 265 E. ovina, 834 s medinensis, 820 Eimeria coccidiosis, 725 Electrophoresis, bacteria and, 184 rapeutic. See Chemothera-Elementary bodies, 67 Elephantiasis, 818 genital, 853, 854 sy to, 410

Ellermann, 880

El Tor cholera, 540	Enzyme(s) (Con
El Tor vibrios, 530, 532	constitutive,
Embden-Meyerhof scheme, 118, 136	induced, 115
EMC viruses, 928	intracellular
Emmonsia crescens, 729	iron-porphyr
E. parva, 729	malic, 134 proteolytic, e
Encephalitis, bovine, 846, 852 chickenpox and, 871	receptor-desi
equine, 943–948	respiratory,
Eastern, 946–947	in bacteria
epidemiology of, 946	Warburg's
vectors of, 947	Enzyme inhibit
Venezuelan, 947–948	folic acid sys
Western, 944-946	indirect, 203
immunity to, 944	limiting syste
pathogenicity for man, 944 vectors of, 945	Eosin-methyler Eperythrozoon
Japanese A, 951	Eperythrozoon
Japanese B, 951-952	E. ovis, 605
pathogenicity for man, 951	E. wenyonii,
Murray Valley, 952-953	Eperythrozoon
Russian spring-summer, 960-962	Epidemic disea
St. Louis, 949–951	Epidemiology,
epidemiology of, 950	305–306
pathogenicity for man, 950 viruses of, 943-949	experimenta of infectious
Encephalitis lethargica, 943	Epidermophyt
Encephalomyelitis, measles and, 900	Equivalence zo
of mice, 920–921	ERC viruses,
of sheep, 962	Erethizon dors
pathogenicity for man, 928	Eretmapodites
porcine, 921	Ergot of rye, 7
Encephalomyocarditis virus(es), 928-	Erosio interdig
929 Endemia disease 202	Erysipelas, 434 immunizatio
Endemic disease, 293 Endocarditis, gonorrheal, 468	swine, 597,
subacute bacterial, 440, 449–450	vaccine for,
vegetative, 598	Erysipeloid, 59
Endolimax nana, 775	Erysipelothrix
Endo's medium, 17	Erysipelothrix
Endotoxins, bacterial, 268	E. monocyte
of Shigella flexneri, 520	E. murisepti
End-product inhibition, 156 Energy, acceptors of, 114	E. porci, 598 E. rhusiopat
biological oxidations and, 112–114	immunity
donors of, 114	morpholo
from tricarboxylic acid cycle, utiliza-	pathogeni
tion of, 129	for mar
Entamoeba coli, 771, 773, 774, 775	E. rhusiopat
E. gingivalis, 775	Erythema ar
E. hartmanni, 773, 774 E. histolytica, 770, 771	602 Erythrasma, 6
epidemiology and control, 774	Erythrocebus
immunity to, 773	Erythromyelol
immunity to, 773 life cycle of, 771	Erythrose-6-pl
E. invadens, 772	Escherich, 483
E. polecki, 775	Escherichia al
Entebbe virus, 960	E. coli, 108
Enteric bacilli, 479–528	144, 159,
classification of, 480 differentiation, biochemical, 483	172, 174, 485, 486,
Enteric orphan viruses, 924–928	596
Enteritis, choleriform, 540	E. coli inter
pneumonia, of calves, 931	E. coli K-12
staphylococcal, 424	E. dispar, 5
Enteritis necroticans, 634	E. dispar va
Enterobacteriaceae, 479–528	E. dispar va
Enterobius vermicularis, 775, 809	E. floccosur
Enterotoxemia, of sheep, 634 Enterotoxin, stanbylococcal, 419	E. freundii, Espundia, 783
Enterotoxin, staphylococcal, 419 Enteroviruses, 913	Esthiomene, 8
shellfish and, 332	Ethers, as disi
Entner-Doudoroff pathway, 121, 136	Eubacteriales,
Entomophthora coronata, 721	Eubacterium,
Envelope of bacteria. See Capsule(s).	Eumycetes, 70
Enzyme(s), adaptive, 236-238	Eumycin, 205
antibody to, 375	European bla
balance of, 245	European rela

Enzyme(s) (Continued)	Evans, 547
constitutive, 115	Evotomys glareolus, 764
induced, 115	Ewe abortion, 846, 852
intracellular localization of, 61–62	Examination, microscopic, 18-21 postmortem, of animals, 25
iron-porphyrin protein, 110 malic, 134	Excreta, water contamination by, 312
proteolytic, exotoxins and, 267	Exoerythrocytic stage of Plasmodium
receptor-destroying, 203, 886	vivax, 784
respiratory, 106	Exotoxins, bacterial, 266
in bacteria, 107–111	proteolytic enzymes and, 267
Warburg's, 110	Expansion, binomial, Soper, 301
Enzyme inhibition, 156, 201 folic acid system, 202	Eye, mycotic infections of, 738 Eye worm, 820
indirect, 203	Djo worm, ozo
limiting systems, 202	
Eosin-methylene blue agar, 17	
Eperythrozoon, 605	F antigens, 535
Eperythrozoon coccoides, 605, 934	F factor, 238
E. ovis, 605 E. wenyonii, 605	FA virus, 921 Farcy, 572, 730
Eperythrozoonosis, 605	Fasciola gigantica, 797
Epidemic disease, 293	F. hepatica, 797
Epidemiology, data of, interpretation,	Fascioloides magna, 797
305–306	Fasciolopsis buski, 797
experimental, 303–305	Fat(s), neutral, 138. See also Lipid(s).
of infectious disease, 293–308 Epidermophytid, 711	Fatigue, resistance and, 284 Fatty acids, oxidation of, 139
Equivalence zone, 365	saturated, 139, 140
ERC viruses, 929	synthesis of, 139
Erethizon dorsatum epixanthum, 964	Favre, 853
Eretmapodites chrysogaster, 967	Feed supplements, antibiotics as, 215
Ergot of rye, 739	Fermentation, 107 alcoholic, 136
Erosio interdigitalis, 716 Erysipelas, 434, 443, 597	bacterial, 135–138
immunization against, 599	biochemical physiology and, 4-5
swine, 597, 598	butyl alcohol, 137
vaccine for, 599	heterolactic, 136
Erysipeloid, 597	lactic acid, 136 lactose, 230
Erysipelothrix, 594 Erysipelothrix erysipeloides, 598	propionic acid, 136
E. monocytogenes, 595	Fernandez reaction, 682
E. muriseptica, 598	Fetus, malformations in, maternal ru-
E. porci, 598	bella and, 902
E. rhusiopathiae, 597–599 immunity to, 599	Feulgen reaction, 57 Fever. See under specific disease names,
morphology and physiology of, 598	as, Andaman A fever.
pathogenicity of, 598	Fibrinolysin, 273
for man, 599	Fibrinolysis, staphylococcal, 421
E. rhusiopathiae suis, 598 Erythema arthriticum epidemicum,	Fibroma, rabbit, 874 Fibroma-myxoma, transduction of, 877
602	transformation of, 242
Erythrasma, 697	Fibromatosis, infectious, 876, 877
Erythrocebus patus, 925	Field fever, 762, 764
Erythromyeloblastosis, 881	Filaria, 818–821
Erythrose-6-phosphate, 122 Escherich, 483	Filariasis, 819 epidemiology and control of, 819
Escherichia alkalescens, 521, 525, 527	Fildes' agar, 579
E. coli, 108, 111, 132, 137, 140, 143,	Filtration, of water, 317
144, 159, 162, 164, 166, 167, 168,	sterilization by, 14
172, 174, 175, 239, 272, 315, 323,	Fimbriae, 45
485, 486, 487, 492, 518, 521, 530,	Fission, simple binary, 81 Fistula of withers, 551
E. coli intermedium, 485	Fixation, autotrophic, 132
E. coli K-12, 524	heterotrophic, 132
E. dispar, 521, 525	of nitrogen, 142
E. dispar var. ceylonensis, 521	Fixed virus, 907
E. dispar var. madampensis, 522 E. floccosum, 716	Flagella, chemical composition, 44 formation of, 223
E. freundii, 485	motion of, 45
Espundia, 783	Flagellates, intestinal, 775-776
Esthiomene, 854	Flagellin, 45
Ethers, as disinfectants, 189	Flagellum(a), 43
Eubacterium 600	Flame cells, 794 Flasks, sterilization of, 13
Eubacterium, 600 Eumycetes, 701	Flavoproteins, 109
Eumycin, 205	Fleming, 204
European blastomycosis, 733	Flexner, 518
European relapsing fever, 749	Flexner bacillus, 516, 526, 527

```
reolus, 764
, 846, 852
microscopic, 18-21
n, of animals, 25
er contamination by, 312
tic stage of Plasmodium
acterial, 266
enzymes and, 267
inomial, Soper, 301
infections of, 738
20
35.
ntica, 797
ı, 797
magna, 797
buski, 797
al, 138. See also Lipid(s).
stance and, 284
```

Flocculation test(s), 31
Kahn, protocol for, 31
Flora, normal, of human body, 288
Fluke(s), blood, human, 798 heterophyid, 798
heterophyid, 798
liver, Chinese, 798
lung, human, 793
Folic acid, bacteria and, 171
Fonsecaea compacta, 717
F. pedrosoi, 717, 718, 722
Fontana stain, for spirochetes, 20
Food, allergy to, 410
preservation of, antibiotics in, 215
Food poisoning, bacterial, 329–331
Clostridium welchii, 332
foodborne infection and, 328-333
Salmonella, 331, 504
staphylococcal, 330
Foot-and-mouth disease, 323, 930-931
Formaldehyde, 190
Forssman antigen, 341, 368
Fort Bragg fever, 764
Foul brood, 614, 621
Fowl pest, pseudo, 897
Fowlpox, 860, 861, 866
Fowl sarcoma, 881
Fracastorius, 1, 663
Frambesia tropica, 757-759
Fränkel, 6, 633
Fränkel's pneumococcus, 453
Freeze-drying, bacteria and, 182
Frei test, 412, 854
Freund adjuvant, 336
Friedländer's bacillus, 485, 487-489,
610
immunological types, 488
pathogenicity of, 488
Frogs, red-leg disease of, 479, 492, 513 Fromme, 763
Frosch, 7, 931
FTA test, 756 Fumarase, 129
Fungi, cultures of, 702
differentiation of genera and species,
705
higher, antibiotics from, 205
hypersensitivity to, 411
microscopic examination of, 702
pathogenic, 700–739
perfect, 701
specimens of, 702
spore formation by, 701
systemic dimorphic, 721–733
Fungi Imperfecti, 701
Fungous ball, 736
Fungous diseases, tissue reactions in,
689
Fusarium roseum, 739
F. sporotrichoides, 739
Fusiformis fragilis, 601
F. fusiformis, 601
F. necrophorus, 601
F. ramosus, 602
Fusobacterium, 600
Fusobacterium plauti-vincenti, 601
Futaki, 765

G-4, 188 G-5, 188 G-11, 188 Gaffky, 495 Gaffkya tetragena, 426 Gallbladder, infection of, 507 Gangrene, 640 gaseous, 623, 631, 636, 637, 639 Garget, 323 Gas, inert, displacement of air by, Gaseous gangrene. See Gangrene. Gastrodiscus hominis, 798 Gastroenteritis, diagnosis of, 502 epidemic nonbacterial, 931 paratyphoid, 505 Salmonella, 504-505 swine, 931 Gay, 368 Gelatin meat extract and meat infusion, Genera of bacteria, 256 Generation, spontaneous, 3-4 Genetic code, 165 Genetics, microbial, 10-11 variation and, 218, 249 Genital tract, resistance and, 286 Geotrichosis, 735 Germicidal equivalent concentration test, 196 Germistan virus, 964 Gestrichum candidum, 735 Getah viruses, 943 Giardia lambia, 775 Gilchrist's disease, 722 Gingivitis, 593 Gingivostomatitis, herpetic, 868 Gland(s). See under specific name. Glanders, 570-575. See also Glanders bacillus. bacterial diagnosis of, 572 chemotherapy of, 573 cutaneous, 572 hypersensitivity in, 411 immunity to, 573 Glanders bacillus, classification of, 571. See also Glanders. morphology and staining, 570 pathogenicity, for lower animals, 571 for man, 572 physiology of, 571 Glandular fever, 873 Glassware, sterilization of, 13 Glomerulonephritis, acute, 448-449 Glossina austeri, 778 G. morsitans, 778 G. pallidipes, 778 G. palpalis, 778 G. swynnertoni, 778 G. tachinoides, 778 Glucose, nonphosphorylating oxidation of, 118 Glucose metabolism, pathways of, importance, 124 Glucose-6-phosphate, formation of, 118 Glycerylphosphorylcholine, 138 Glycine, biosynthesis of, 150 Glycolysis, phosphorylating, 118 Glyoxylic acid cycle, 129 Goats, abortion in, 550 brucellosis in, 550 contagious agalactia of, 608 Golgi, 783 Gonidia, formation of, 82 Gonococcus(i), 463-470 antigenic structure of, 467 drug resistance of, 466 immunity to, 469 infections with, bacteriological diagnosis of, 469 chemotherapy for, 468 morphology and staining, 463 pathogenicity, for lower animals, 469 for man, 467 physiology of, 464 toxins of, 466 variations in, 466

incidence of, 467 Grahamella talpae, 605 Gram stain, 19, 47 procedure for, 20 Gramicidin, 205 Gramicidin S, 205 Granules, Babes-Ernst, 59 cytoplasmic, 59 Granuloma, Candida, 716 coccidioidal, 725 paracoccidioidal, 729 swimming pool, 683 Granuloma inguinale, 610, 853 Granuloma venereum, 610 Grassi, 783 Gray patch, 708 Greenwood binomial expansion, 301 Greig test, 533 Grippe, Balkan, 842 Ground itch, 813 Growth, of bacteria, 81-86 exponential growth phase, 85 lag phase, 84 logarithmic phase, 85 of microorganisms, 81-102 physiology of, comparative, 101-102 Growth factor, 168 Growth mediums for tissues, 28 Gruby, 687, 714 Guarnieri bodies, 67, 860, 866 Guaroa virus, 964 Guinea pig, anaphylactic shock in, 405 Guinea worm, 820 Gymnoascus monbreunii, 730 Gyppy tummy, 525 H agglutinins, 511 H antigens, 346, 486, 496, 497, 511, 535 HA 2 virus, 894 Haemagogus capricornii, 959 H. equinis, 959 H. lucifer, 960 H. spegazzini, 959 H. spegazzini falco, 959, 960 Haemaphysalis bispinosa, 842 H. cinnabarina, 569 H. concinna, 839, 962 H. humerosa, 842 H. leachii, 839 H. leporis palustris, 569, 838 Haemobartonella, 605 Haemobartonella bovis, 605 H. canis, 605 H. microtii, 605 H. muris, 605 H. tyzzeri, 605 Hajna bismuth sulfite medium, 17 Hank's BSS, 27 Hansen, 678 Hansen's bacillus, 663, 678-682 Haptenes, 336, 344 in anaphylaxis, function of, 406 precipitin reactions with, 367 Harvest fever, 762, 764 Hasami fever, 765 Haverhill fever, 602-603 Haverhillia multiformis, 602, 603 Hay fever, 409 Heat, lethal, 179 moist, 179 sterilization by, 180 Heel, cracked, 697 Heine, 914

Heine-Medin disease, 914

HeLa cell culture, 28

Gonorrhea, diagnosis of, 469 epidemiology of, 468

Hellriegel, 5	Herpes (Continued)	Hydrogen (Continued)
Helvella esculenta, 739	H. progenitalis, 869	transfer of, 106
Hemadsorption, 886	H. simplex, 867–870	Hydrogen sulfide, Brucella and, 548
Hemadsorption viruses, 893	chemotherapy of, 869	Hydrolysis, of complex sugars, 115
Hemagglutination, 886	experimental infections of, 869	Hydrophobia. See Rabies.
passive, 368	immunity to, 869	Hygrophorus conicus, 739
Hemagglutination-inhibition test, 886	pathogenicity for man, 867	Hymenolepsis diminuta, 806
Hemagglutinins, 367, 888	primary, 868	H. fraterna, 806
Hemoflagellates, 776–783	recurrent, 868	H. nana, 805, 806
Hemolysins, 270 filterable, 270	H. tonsurans, 709 H. zoster, 871–872	Hypersensitivity, 404–412. See also Allergy.
filtration of, protocol for, 33	Herpesvirus(es), 73, 867–872	anaphylaxis and, 404–408
of cholera vibrio, 535	replication of, 96	immunity and, 412
virulence and, 271	Herpesvirus hominis, 867	in infection, 411–412
Hemolysis, 377	Heterofermenter, 136	streptococcal, 433
blood-plate, 271	Heterograff, 340	to horse serum, 408
Hemophilic bacteria, 578-587	Heterophyes heterophyes, 797	Hypochlorite, in water purification,
Hemophilus aegyptius, 582	Heterotrophs, 248	319
H. bovis, 581	Hexachlorophene, 188	
H. canis, 580, 581	Hexokinase, 118	
H. ducreyi, 586–587	Hexoses, breakdown of, 117–125	Too hostonia in 212
H. duplex, 586	Highlands I viruses, 943	Ice, bacteria in, 313 Idiosyncrasy, drug, 410
H. gallinarium, 581 H. hemoglobinophilus, 580	Hikojima antigens, 535 Hinton test, 755	IgA, 349, 351, 397, 411
H. hemoglobinophilus canis, 580	Hippelates pallipes, 758	IgD, 349
H. hemolyticus, 580	Hiss-Russell Y bacillus, 516	IgG, 349, 351, 403
H. influenzae, 578-581, 582, 891.	Hiss's capsule stain, 20	IgM, 349, 351
See also Influenza and Influenza	Histamine, shock and, 406	Ilesha virus, 964
virus(es).	Histidine, biosynthesis of, 150	Ilheus virus, 957
antigenic structure of, 580	Histomonas meleagridis, 775	Immune response, 401
carriers of, 580	Histoplasma capsulatum, 412, 689, 721,	Immune state, 388–412
chemotherapy of, 581	730, 732, 733	Immunity, 8–9, 335–362, 364–385
morphology and staining, 578	pathogenicity for lower animals, 732	acquired, 399–412
pathogenicity, for lower animals,	H. duboisii, 723, 730	active, 399–403 antitoxic, 389
581 for man, 580	H. farciminosum, 730 H. uveitis, 738	cellular, 390–397
physiology of, 579	Histoplasmin, 412, 732	response in, 392
toxins of, 580	Histoplasmosis, 730	herd, 302
varieties of, 580	diagnosis of, 733	humoral, 388-390
H. influenzae suis, 580, 891	epidemiology for, 732	hypersensitivity and, 412
H. muris, 581	pathogenicity for man, 731	local, 396
H. ovis, 581	Hives, 409	natural, 397–399
H. parainfluenzae, 579-580	Hofmann's bacillus, 660	specific, 397
H. parapertussis, 583 H. pertussis, 555, 582–586. See also	Holmes, 5 Homofermenter, 136	passive, 403 phagocytosis and, 389
Whooping cough.	Homograft reactions, antigens and, 340	thymus and, 402
antigenic structure of, 583	Hookworm(s), 812	to actinomycosis, 695
morphology and staining, 582	diagnosis of, 813	to African sleeping sickness, 778
pathogenicity, for animals, 584	epidemiology and control of, 814	to anthrax, 619
for man, 583	immunity to, 813	to bacillary dysentery, 527
physiology of, 582	Hormodendrum pedrosoi, 717	to Bartonella bacilliformis, 605
toxins of, 583	Hormones, adrenocortical, resistance	to botulism, 646
H. suis, 262, 580 H. vaginalis, 587	and, 287 imbalance of, infection and, 287	to brucellosis, 554 to Chagas' disease, 780
Hemorrhagic fever, 955	Horses, brucellosis of, 551	to cholera, 541
Hepatitis, canine, 934	Horse serum, hypersensitivity to, 408	to Coccidioides immitis, 727
enzootic, 966	Host, resistance of, 277–289	to Colorado tick fever, 964
infectious, 932-933	toxicity and, 275-277	to dengue, 955
necrotic, 638	Host population(s), 298-300	to dermatophytes, 711
murine, 934	parasite populations and, interaction,	to diphtheria, 656
of lower animals, 934–935	300–303	to Entamoeba histolytica, 773
serum, 933–934	Hotis test, 323	to glanders, 573
viral, 932–935 Hepatitis viruses, 913	Hoyle's medium, 650 Hübener, 763	to gonococci, 469
Hepatitis virus A, 933	Hucker ammonium oxalate crystal vio-	to herpes simplex, 869 to hookworm, 813
Hepatitis virus B, 933	let stain, 19	to influenza, 892
Herd immunity, 302	Humoral immunity, 388–390	to kala-azar, 781
Heredity, allergy and, 409	Hunter, 753	to leprosy, 681
blood group antigens and, 340	HVJ virus, 893	to lymphocytic choriomeningitis, 970
resistance and, 279	Hyaluronidase, 274	to lymphogranuloma venereum, 854
rheumatic fever and, 448	staphylococcal, 421	to malaria, 787
tuberculosis and, 670	streptococcal, 432	to measles, 900
d'Herelle, 7	Hydatid disease 805	to meningococci, 476
Herpangina, 868, 923 Herpes circinatus, 709	Hydatid disease, 805 Hydrogen, acceptor of, 106	to mumps, 896 to Newcastle disease virus, 898
H. desquamans, 709	donor of, 106	to North American blastomycosis,
H. febrilis, 867	liberation with decomposition of	724
H. labialis, 867	amino acids, 145	to plague, 566
	egil karala kabupatèn Kabupatèn Kabupatèn K	

	Immunity (Continued)	Infantile paralysis, 914. See also Polio-
	to pneumococci, 461	myelitis.
	to poliomyelitis, 919	Infection(s), airborne, 295
	to psittacosis, 852	cutaneous, 704-712
	to rabies, 908	foodborne, bacterial, 331–332
	to relapsing fever, 748	control of, 333
	to rhinoviruses, 930	food poisoning and, 328–333
	to rickettsia, 830	parasitic, 333 gallbladder, 507
	to rubella, 902 to Russian spring-summer encepha-	gonococcal, chemotherapy for, 469
	litis, 961	diagnosis of, bacteriological, 469
	to schistosomes, 801	hormonal imbalance and, 287
	to smallpox, 863	hypersensitivity in, 411-412
	to sporotrichosis, 720	inapparent, 398
	to staphylococci, 425	meningococcal, diagnosis of, bacteri-
	to streptococci, 441	ological, 475
	to syphilis, 754	multiple, interference and, 99-101
	to Taenia solium, 804	of bacterium, 88
	to tetanus, 628	of mucous membranes, 715
	to toxoplasmosis, 792	pinworm, 809
	to tuberculosis, 672	pneumococcal, diagnosis of, bacteri-
	mechanism of, 674	ological, 460 Rachmat, 765
	to tularemia, 569 to typhoid fever, 510	Salinem, 765
	to WEE virus, 944	Salmonella, 503–513
	to Weil's disease, 763	diagnosis of, bacteriological, 502
	to whooping cough, 585	secondary, chemotherapeutic drugs
	to yellow fever, 960	and, 211
	Immunization, aerosol, 396	staphylococcal, hospital-acquired,
	against anthrax, 620	422
	against botulism, 646	prevalence of, 424
	against Clostridium chauvoei, 642	suppurative, 423
	against diphtheria, 657	streptococcal, diagnosis of, bacteri-
	against erysipelas, 599	ological, 443
	against influenza, 892	immunity to, 441 of skin and subcutaneous tissues,
	against measles, 900 against plague, 566	443-444
	against pneumococci, 461	of upper respiratory tract, 444-445
	against spotted fever, 839	wound, 444
	against staphylococci, active, 426	Infection pressure, 303
	against tetanus, active, 629	Infection prevention tests, 196
	passive, 629	Infectious bulbar paralysis, 870
	against tsutsugamushi, 840	Infectious disease, control of, 307–308
	against tuberculosis, active, 675	incidence of, analysis, 301
	against tularemia, 570	Infectious jaundice, 763
	against typhoid fever, 512 passive, 513	Infectious mononucleosis, 873 Inflammation, body cells and, 392
	against typhus, 837	Influenza, 886. See also Hemophilus
	against whooping cough, 585	influenzae and Influenza virus(es).
	in disease control, 308	immunity to, 892
	precocious, 403	immunization against, 892
	vaccinia and, 864	laboratory diagnosis of, 892
	with killed bacteria, 400	pneumonia and, 892
	with microorganisms, living attenu-	swine, 891
	ated, 400	Influenza A, 887, 888, 890
•:	living virulent, 399	Influenza B, 887, 888, 889
	with products of microorganisms,	Influenza C, 888
	Immunodiffusion, 365	Influenza virus(es), 886-892. See also Hemophilus and Influenza.
	Immunoelectrophoresis, 366	avian, 891
	Immunoglobulin(s), 348	experimental infections with, 891
	7S, 348	morphology of, 887
	198, 348	pathogenicity, for lower animals, 891
	biological activity of, 350	for man, 891
	classes of, 349	replication of, 94
	degradation of, 349	toxin of, 887
	properties of, 349	variation in, 889
	Immunological methods, 29–35	antigenic, 888, 889
	Imvic reactions, 485 Inaba antigens, 535	Inhibition, enzyme. See Enzyme inhibi-
	Inada, 763	tion. Initial bodies, 67
	Inclusion blennorrhea, 855, 856	Inoculation, against whooping cough,
	Inclusion bodies, 67	585
	Inclusion conjunctivitis, 846, 856	animal, 24-25
	Incompatibility, tissue, 340	routes of, 24
	Indian tick typhus, 839	intracutaneous, 24
	Indol, formation from tryptophan, 484	Insect(s), as vectors, control of, 307
	Induction, mutation vs., 247	of brucellosis, 553

olio- Insect(s) Continued) of tularemia, 569 intramuscular, 24 intraperitoneal, 24 intravenous, 24 prophylactic, scarlet fever, 446 typhoid fever, 512 subcutaneous, 24 Inocybe infelix, 739 I. infida, 739 Insect viruses, 75-76 Instruments, sterilization of, 13 Interference between viruses, 99-101 Interferon, 101 International Unit, defined, 210 Intestinal flagellates, 775-776 Intestinal tract, resistance and, 286 Invasin, 274, 440 Iodamoeba bütschlii, 775 Iodine, water purification by, 319 Iodinin, 205 Irradiation. See Radiation. Iso-antigens, 341 Isocitratase, 129 Isocitric dehydrogenases, 129 Isolation, in disease control, 307 rugs of pathogenic milk bacteria, 326 Isoleucine, 149 Isoodon macrourus, 842 Isospora, 725 Isospora belli, 791 I. hominis, 791 teri- Itch, Cuban, 862 ground, 813 mad, 867, 870 sues, miner's, 813 swimmer's, 802 -445 Ito, 763 Iwanowski, 7 Ixodes holocyclus, 839, 842 I. persulcatus, 962 I. ricinus, 962 I. ricinus californicus, 569

> Jantigens, 535 Jäger, 470 Jail fever, 835 Japanese A encephalitis, 951 Japanese B encephalitis, 951-952 Japanese flood fever, 840 Japanese seven-day fever, 765 Jaundice, epidemic, 932 infectious, 763 postvaccinal, 933 Jaw, lumpy. See Actinomycosis. Jenner, 8, 864 Johne's bacillus, 411, 663, 682 Johne's disease, 682 Johnin, 411 Johnin reaction, 682 Junin virus, 955

K antigens, 346, 846
Kaffir pox, 862
Kagami fever, 873
Kahn test, 755
leprosy and, 682
protocol for, 31
Kairi virus, 964
Kaisodi virus, 960
Kala-azar, 781
diagnosis and treatment of, 781
epidemiology and control, 782
immunity to, 781

Kaposi's varicelliform eruption, 868	Lactobacillus (Continued)	Leptospirosis (Continued)
Karyosome, 771	L. delbreuckii, 111, 591	chemotherapy of, 763
Kauffmann-White schema, 497	L. fermenti, 591	laboratory diagnosis of, 762
Kedani fever, 840	L. helveticus, 591	Lethal dose, 264
Kefir, 324 Kalaid blastomygasis, 729	L. lactis, 591 L. leichmannii, 591	Leucine, biosynthesis of, 149 Leucocidin(s), 272, 419
Keloid blastomycosis, 729 Keloid formation, 708	L. pastorianus, 591	Neisser-Wechsberg, 419
Kemerovo virus, 960	L. plantarum, 591	Panton-Valentine, 419
Kenya fever, 839	L. thermophilus, 591	pneumococcal, 455
Keratitis of lens, Aspergillus and, 737	Lactose, fermentation of, 230	Leucolysin, 419
Keratoconjunctivitis, epidemic, 937	Landsteiner, 914	Leuconostoc, 456
herpetic, 868	Lane diagnosis of hookworm, 813	Leuconostoc delbrueckii, 126
Keratolysis, pitted, 697	Langat virus, 960	L. mesenteroides, 116, 126, 174
Keratolysis plantare sulcatum, 697	Langenbeck, 6, 687, 714	Leucosis, avian, 880, 881
Kerbert, 793	Large-Sachs dysentery bacilli, 517, 518	erythromyeloblastic, 881
Kerion, 708	Laurer's canal, 794	lymphomatosis, 881
Kermack and McKendrick Threshold	Laveran, 783	Leukemia, mouse, 879
Theorem, 301, 302	Laveran-Mesnil reactions, 371	Levaditi phenomenon, 371
Kernig's sign, 916	Lavington I bacillus, 520	Lewis, 771
α-Ketoglutaric acid oxidase, 129	Laybourn Albert's diphtheria stain, 19	Licheniformin, 205
Kinases, bacterial, 273	L.C.L. bodies, 67, 850	Liebig, 4
Kissing disease, 873	LD ₅₀ , 264	Life cycles, microbial, 225
Kitasato, 6, 8, 373, 559, 624	Lead acetate agar, preparation of, 15	Ligniers, 574
Kitt, 570 Klebs, 6, 649	Lecithinase D, 139 Lecithins, 138	Limberneck in chickens, 330 Lipid(s), 345. See also Fat(s).
Klebs-Löffler bacillus, 649	Lecithovitellin, 635	hydrolysis of, 138
Klebsiella ozaenae, 488	van Leeuwenhoek, Antony, 1	metabolism of, 138–140
K. pneumoniae, 485, 487-489. See	Leishman, 781	α-Lipoic acid, 126
also Friedländer's bacillus.	Leishmanias, 781–783	Lipovnik, 960
K. rhinoscleromatis, 488	Leishmania braziliense, 776, 783	Lister, 5
Kleine, 777	L. chagasi, 782	Listerella hepatolytica, 594
Kligler, 574	L. donovani, 776, 781, 782, 783	L. monocytogenes, 594
Kligler's iron agar, 502	life cycle, 781	Listeria, 594
Kline test, 755	L. infantum, 782	Listeria bovina, 596
Koch, 5, 6, 530, 536, 541, 581, 597, 614,	L. tropica, 776, 783	L. cuniculi, 596
631, 663, 687	Leishmaniasis, cutaneous, 783	L. gallinarum, 596
Koch phenomenon, 412, 673	mucocutaneous, 783	L. gerbilli, 596
Koch's postulates, 259–262	visceral. See Kala-azar.	L. monocytogenes, 594–597, 598, 873
Koch-Weeks bacillus, 581–582	Leontocebus geoffroyi, 734	antigenic structure, 596
Koliba virus, 960	Lepiota morgani, 739	morphology and staining, 595
Kolmer test, 755	Lepra cells, 678	pathogenicity of, 596
Koongal virus, 965	Leproma, 679 Lepromin, 411, 682	physiology of, 595
Koplik spots, 899 Koumiss, 324	Leprosy, 678-682. See also Mycobac-	toxins of, 596 L. ovis, 596
Kracke blood culture medium, 16	terium leprae.	Listeriosis, 596
Krebs tricarboxylic acid cycle, 128-131	chemotherapy of, 681	epidemiology of, 597
as source of intermediates, 129	experimental infections of, 679	Litomosoides carinii, 820
utilization of energy from, 129	hypersensitivity in, 411	Liver fluke, Chinese, 798
KRP test, 755	immunity to, 681	Liver rot, 797
Kruse, 517	Kahn test and, 682	Loa loa, 818, 820
Küchenmeister, 803	nerve, 679	Loboa loboi, 689, 730
Kumba virus, 948	pathogenicity of, 679	Lobo's disease, 730
Kurthia zenkeri, 490	rat, 682	Locke's solution, 186
Kyasanur Forest disease, 962-963	transmission of, 680	Lockjaw. See Tetanus.
	Wassermann test and, 682	Löffler, 6, 7, 570, 597, 649, 931
	Leptomeningitis, 854	Löffler's alkaline methylene blue, 19
T antigens 246 496 960	Leptospira(s), 760–765	Löffler's medium, 16, 650
L antigens, 346, 486, 860 L bodies, 223	antigenic structure of, 761	Looss, 812 Louping ill, 962
Laboratory methods, 13–35	cultivation of, 760 morphology of, 760	Löwenstein's medium, 665
Lactic acid, fermentation of, 136	pathogenicity of, 761	LS antigen, 860
Lactobacillaceae, 600	saprophytic, 765	Lucretius, 1
Lactobacillus(i), 590-594	Leptospira akiyami A, 765	Lumbo virus, 965
classification of, 591	Lept. australis A, 765	Lumpy jaw. See Actinomycosis.
dental caries and, 593	Lept. australis B, 765	Lumpy wool, 698
heterofermentative, 591	Lept. autumnalis, 764, 765	Lung fluke, human, 793
homofermentative, 591	Lept. ballum, 764	Lupus erythematosus, 669
morphology of, 590	Lept. bataviae, 765	Lutz's disease, 729
physiology of, 591	Lept. biflexia, 761, 765	Lygranum, 412, 855
saliva and, 594	Lept. canicola, 764, 765	Lymphadenitis nonbacterial regional
Lactobacillus acidophilus, 323, 591, 592	Lept. grippotyphosa, 764, 765	[872]
L. arabinosus, 171, 172, 173, 175	Lept. hebdomadis, 765	Lymphocytic choriomeningitis, 969-
L. bifidus, 591, 592	Lept. icterohemorrhagiae, 763, 764	971
L. brevis, 136, 591	Lept. interrogans, 761	epidemiology of, 970
L. buchneri, 591	Lept. pomona, 764, 765	immunity to, 970
		THE RESERVE OF THE PROPERTY OF
L. bulgaricus, 171, 323, 591, 592	Lept. pyrogenes, 765	pathogenicity for man, 970
		pathogenicity for man, 970 Lymphocytopoiesis, 402 Lymphogranuloma inguinale, 610, 85:

Marchiafava, 470

Marcy, 931

Martini's equations for immunizing Meningopneumonitis, 852 Lymphogranuloma venereum, 412, 853chemotherapy of, 849 diseases, 303 855 Mary Mallon (Typhoid Mary), 510 opossum, 852 epidemiology of, 855 Meriones libycus, 563 immunity to, 854 Mastigophora, 775-783 Mastitis, bovine, 323, 558 staphylococcal, 425 morphology and staining, 846 pathogenicity, for lower animals, M. persicus, 563 M. tristrami, 563 M. vionogradovi, 563 Maurer's dots, 786 for man, 853 Maximov, 9 Merozoites, 784 toxin of, 847 . Mesophiles, 178 Mayaro virus, 948, 949 Lymphogranuloma venereum-psittaco-Measles, 898-901 Mestor megista, 780 sis organisms, 66-67 encephalomyelitis and, 900 Metabolism, bacterial, 105-176 experimental infections with, 901 Lymphopathia venereum, 853 carbohydrate, 114-138 Lymphoreticulosis, benign inoculation, German, 901-903 glucose, pathways of, importance, 124 872 immunity to, 900 inorganic nitrogen, 140-142 immunization against, 900 Lysine, 148 lipid, 138 Lysins, 376-381 pathogenicity for man, 899 nitrate, 140 Meat extract and meat infusion gelatin, hot-cold, 270 of amino acids, 142-157 Lysis, hot-cold, 270 by obligate anaerobes, 145 of nucleic acids, 158-163 mechanism of, 379 Meat extract broth, agar and, 15 Meat infusion broth, agar and, 15 of nucleotides, 158-163 Lysogeny, 90 Lysol, 188 Medical mycology, 687-739 of proteins, 142-157 Lysolecithin, 138 Medical parasitology, 769-821 of purines, 161-163 Lysolecithinase, 138 Medin, 914, 918 of pyrimidines, 161-163 Medium(s), 27, 199 of pyruvic acid, coenzymes of, 125 Anderson's, 650 Metacercaria, 794 Clauberg, 650 Metagonimus yokogawai, 798 M antigen, 436, 440, 441, 442, 549 Corper's, 665 Metal-organic compounds, as disinfecculture. See Culture medium(s). Czapek-Dox, 702 tants, 187 Metals, disinfecting actions of, 186 M colony, 221 M-25 virus, 895 Macaca cynomolgus, 901 deoxycholate-citrate, 17 Metazoa, 793-821 M. mulatta, 420 Metchnikoff, 9, 390, 592, 752 Dieudonné, 538 M. radiata, 963 growth, 28 Methionine, 148 M. rhesus, 901 Hoyle's, 650 Methyl red test, 484 Löffler's, 650 Machado reaction, 780 Methylene blue reduction of milk, Machupo virus, 955 Löwenstein's, 665 326 Macrogamete, 784 maintenance, 28 Mice. See Mouse. Macrogametocyte, 784 Neill's, 650 Microbacterium, 594 Macrophages, 390 Mad itch, 867, 870 Microbacterium flavum, 594 Petragnani's, 665 Trudeau, 665 M. lacticum, 594 Medlar, 717 Mehlis' gland, 794 Microbial genetics, 10-11 Madura foot, 698 Microbial life cycles, 225 Madurella, 700 Maduromycosis, 698-700 Meischer's tubes, 792 Microbial phylogeny, 247-249 Aspergillus and, 737 Melao virus, 965 Microbial population, 297-298 "Magic bullet," 197 Melioidosis, 573 Microbial toxins, 266-277 Maintenance mediums for tissues, 28 Melitin, 411 tissues and, 277 Makonde virus, 948 Melophagus ovinus, 825 Microbial variations, genetics and, 218-Mal de caderas, 907 Membrane filter procedure for testing 249 Mal de pinto, 759-760 nature of, 243-247 water, 314 Maladie de Nicolas et Favre, 853 Malaria, 787, 783. See also *Plasmodium*. Mengo virus, 928 Microbial virulence, 263-277 Meningitis, aseptic, 896, 924, 926 Microbiology, historical development benign tertian, 787 aspergillar, 736 of, 1-12 chemotherapy of, 788 cryptococcus, 733 of food, 328-333 diagnosis of, 788 epidemic, cerebrospinal, 470 of sewage, 319-320 epidemiology and control, 789 eosinophilic, 815 of water, 310-319 estivo-autumnal, 787 α-hemolytic streptococcal, 440 Micrococcus(i), 426 falciparum, 787 Hemophilus influenzae and, 580 shape of, 39 immunity to, 787 meningococcal, 473 epidemics of, 474 Micrococcus gonococcus, 463 malignant tertian, 787 M. intracellularis meningitidis, 470 pernicious, 787 pneumococcal, 460 M. lanceolatus, 453 tuberculosis, 669 quartan, 787 M. lysodeikticus, 285 Malate synthetase, 131 Meningococcemia, 473 M. melitensis, 547 Malic dehydrogenases, 129 Meningococcus(i), 470-477 M. meningitidis, 470 Malic enzyme, 134 carriers of, 473 M. pneumoniae, 453 Malignant pustule, 618 chemotherapy for, 475 M. pyogenes var. aureus, 415 Mallassezia furfur, 703 classification of, 472 M. tetragenus, 426 Mallein, 411 epidemiology of, 473 Microfilaria, 818 Mallein test for glanders, 572 immunity to, 475 Microgamete, 784 Malnutrition, resistance and, 283 infection with, bacteriological diag-Microgametocyte, 784 Mammals, psittacosis in, 851 nosis of, 475 Micromonospora keratolytica, 697 Manceaux, 791 morphology and staining, 470 Micromys minutus, 765 Manchurian typhus, 836 pathogenicity, for lower animals, 475 M. soricinus, 765 Mansom, 783 for man, 472 Microorganism(s), adaptation of, 228. Mansonella ozzardi, 818, 820 See also Bacteria. physiology of, 471 Mansonia perturbans, 947 toxins of, 472 antigenic structure of, 345-347 variations in, 472 M. titillans, 947 antigenic variation of, 347 Mantoux test, 674 Meningoencephalitis, 896 attenuation of, 228

eosinophilic, 815

herpetic, 868

Bergey classification of, 254-255

cellular response to, 288

Microorganism(s) (Continued) Milk (Continued) Mycetoma (Continued) characterization of, immunological, plate counts of, 325 laboratory diagnosis of, 700 quality of, determination of, 325 mycotic, 700 development of alternate metabolic regulation of, 328 Mycid, 711 Mycobacterium(a), 663-683 "anonymous," 682 red, 324 pathways in, 235 sediment test of, 326 drug-dependent, 234 "cold-blooded," 683 growth of, 81-102 streptococcal infection from, 445 killed, immunization with, 400 yellow, 324 Mycobacterium avium, 663 Milk products, 328 Myco, balnei, 683 living attenuated, as antigens, 336 immunization with, 400 Miner's itch, 813 Myco. butyricum, 683 living virulent, immunization with, 399 Miracidium, 794 Myco. chelonei, 683 Mite typhus, 840-841 Myco, fortuitum, 683 Myco. intracellularis, 683 morphology of, comparative, 78 Mitsuda reaction, 682 pathogenic, disease and, 259-289 MM virus, 928 Myco. kansasii, 683 phylogeny of, 247-249 Modoc virus, 949 Myco. leprae, 663, 678-682. See also physical and chemical structure of, Molluscum contagiosum, 875-876 Leprosy. cultivation of, 679 36-78 Moloney test, 658 Monilia, 714-716 morphology and staining, 678 products of, immunization with, 400 psittacosis-lymphogranuloma vener-Monkey kidney tissue culture, 28 Myco. leprae murium, 663, 682 eum group, 846-857 Monocytosis, 596 Myco. luciflavum, 683 antigenicity of, 848 Mononucleosis, infectious, 873 Myco. marinum, 683 resistance of, cross, 234 Monoxygenases, 110 Myco. paratuberculosis, 411, 663, 682 induced, 233 Montenegro reaction, 783 Myco. phlei, 683 Morax-Axenfeld diplobacillus, 586 Myco. piscium, 683 origin, 232 physiology of, 235 Morbus gallicus, 751 Myco. ranae, 683 Morgan's bacillus, 491, 492 Myco. scrofulaceum, 683 to chemotherapeutic agents, 231-Mosaic, antigenic, 369 Myco. smegmatis, 683 236 Myco. thamnopheos, 683 transfer of, 233 Moscow typhus, 836 selection of environment for, 227 Mosquito-borne viruses, 965-967 Myco. tuberculosis, 694 Mossman fever, 762, 765 taxonomy of, 252-258 uniqueness of, 218-220 Myco. tuberculosis var. bovis, 663 Mossuril virus, 965 Myco. tuberculosis var. hominis, 663variation of, biochemical, 229-231 Motility, microscopic demonstration of, genetics and, 218-249 Myco. tuberculosis var. muris, 682 morphological, 220-227 Mountain tick fever, 963 Myco. ulcerans, 683 nature of, 243-247 observed, 220-243 Mouse(ice), encephalomyelitis of, 920-Mycocerosic acid, 666 Mycolic acid, 666 physiological, 227-236 neoplastic diseases of, 879-880 Mycology, medical, 687-739 Mycoplasma, 605-610 Microphages, 390 septicemia of, 597 typhoid in, 304, 504 morphology and staining, 606 Microscope, compound, 38 darkfield, 3, 38 Mousepox, 304, 861, 864, 866 of man, 609-610 Mouth, resistance and, 286 physiology of, 607 electron, 3, 38 reproduction in, 82 first, 2 spirochetes of, 750 phase, 38 varieties, strains and species, 607 Much, 664 Microscopy, 1-3, 37-39 Microsporid, 711 Much granules, 664 Mycoplasma fermentans, 609, 610 Mucin, virulence and, 265 M. hominis, 609, 610 Microsporum audouini, 708 Mucinase, of cholera vibrio, 535 M. mycoides, 606 M. orale, 609 M. canis, 708 Muco-antibody, 397 M. fulvum, 710 Mucor, 701 M. pharyngis, 609 Mucormycosis, 737 M. pneumoniae, 609, 610 M. gypseum, 702, 709, 710 M. salivarum, 609 M. minutissimum, 697 Mucous membranes, infections of, 715 M. nanum, 709 Mud fever, 764 Mycoplasmatales, 608 Microtus agrestis, 682 Müller, 2 Mycoses, cutaneous, 704-712 M. arvalis, 764 Mumps, 895-898 subcutaneous, 717-721 M. montebelli, 765, 841 experimental infections with, 897 superficial, 703-704 Middleburg viruses, 943 immunity to, 896 systemic, 721-738 Milk, as culture medium, 15 Mycotoxicosis, 738-739 orchitis and, 896 Myocarditis, 924 bacteria in, sources of, 322 pancreatitis and, 896 external, 323 pathogenicity for man, 896 diphtheritic, 655 pathogenic, 324 Muriviruses, 929 rheumatic, 447 isolation of, 326 Murray Valley encephalitis, 952-953 Myxoma, rabbit, 874 Mus concolor, 841 bitter, 324 Myxoma-fibroma, transduction of, 877 blue, 324 M. diardii, 841 transformation of, 242 brucellosis and, 552 M. musculus, 840 Myxomatosis, infectious, of rabbits, 876 cell count of, 326 Muscarin, 739 Myxoviruses, 72, 885-886 certified, 326 Musgrave, 518 coliform count of, 326 Mushroom poisoning, 738 fresh, bactericidal property of, 325 Mutation, 243-245 Grade A, 325 Grade B, 325 induction vs., 247 Nabarro, 777 spontaneous, 243 Nagana, 781 hygienic control of, 326 Muton, 238 Nägeli, 2 inspection of, 326 Myalgia, epidemic, 923 Nagler reaction, 635 lactic acid, 323 Mycetismus cerebis, 739 Nairobi sheep disease virus, 960 methylene blue reduction of, 326 M. choleriformis, 739 Nanivirus, 913 microbiology of, 322-328 M. gastrointestinalis, 738 Nannizzia gypsea, 702 microscopic counts of, 325 M. nervosus, 739 N. incurvata, 702 mother's, antibody in, 403 M. sanguinareus, 739 Nanukayami, 765 pasteurization of, 327 Mycetoma, 696, 698-700 Nasopharynx, resistance and, 285 phosphatase test for, 327 actinomycotic, 700 Ndumu viruses, 943

Necator americanus, 812, 813, 814, life cycle of, 812 Necrotoxin, 275 Needham, 3 Needles, sterilization of, 13 Negishi virus, 960 Negri bodies, 67, 904, 907 Neill's medium, 650 Neisser-Wechsberg leucocidin, 419 Neisser-Wechsberg phenomenon, 379 Neisseria, 463-477 Neisseria catarrhalis, 476 N. flava, 477 N. gonorrhoeae, 463 N. intracellularis, 470 N. meningitidis, 470 N. perflava, 477 N. sicca, 477 Nematoda, 808-821 Nematospiroides dubius, 815 Nephritis, acute interstitial, 655 streptococcal, 445 Neuralgia, herpes zoster and, 872 Neuritis, cranial, chickenpox and, 871 Neurodermatitis, 409 Neurosyphilis, 754 Neutralization test, 266 Newborn, tetanus of, 628 Newborn pneumonitis virus, 893 Newcastle disease virus, 895, 897-898 experimental infections with, 898 immunity to, 898 pathogenicity for man, 898 Newcastle-Manchester bacilli, 517, 519 Niacin, bacteria and, 169 Nicolaier, 624 Nicolas, 853 Nicolle, 791 Nicotinamide-adenine dinucleotide (NAD), 108 Nicotinamide-adenine dinucleotide phosphate (NADP), 108 Nicotinic acid, bacteria and, 169 Nippostrongylus brasiliensis (muris), 815 Nitavirus. See Herpes virus(es). Nitrate, metabolism of, 140 Nitrate broth, preparation of, 15 Nitrate reductase, 141 Nitrification, 141 Nitrite reductase, 141 Nitrofurans, 200 Nitrogen, fixation of, 142 inorganic, metabolism of, 140 Nitrosomonas europaea, 107 Njovera, 757 Nocardia astroides, 689, 695 N. brasiliensis, 696, 698, 700 N. caviae, 696 N. farcinica, 696 N. intracellularis, 683 Nocardin, 696 Nocardiosis, 695-697 Nocard's bacillus, 850 Nodules, Aschoff, 447 Noguchi, 746, 751 Nomenclature, bacterial, 256 Nonexanthematous tick fever, 963 North Queensland tick typhus, 839 Nose, resistance and, 285 Novy, 637 NP antigen, 860 Ntaya virus, 956 Nucleic acid(s), breakdown of, 159 infectious, 97 metabolism of, 158-163 synthesis of, 163-167

Nucleodepolymerases, 159
Nucleoproteins, 159
Nucleosidase, 161
Nucleosides, 159
Nucleotidase, 161
Nucleotidase, 161
Nucleotides, metabolism of, 158–163
Nucleus, bacterial, 57
Nutrition, of bacteria, 168–176
resistance and, 283
Nuttall, 376, 633
Nyamanini virus, 960
Nyando virus, 965

O agglutinins, 511 O antigens, 346, 486, 496, 497, 511, 535, Obermeier, 745 Ogawa antigens, 535 O'Hara's disease, 569 Oidium albicans, 714 Old tuberculin (OT), 673 Oligosaccharides, 115 Omsk hemorrhagic fever, 963 Onchocerca volvulus, 818, 820 Onion, black streak of, 738 Ontogeny, 401 Onychomycosis, 716, 738 Onychomys torridus, 727 O'nyong-nyong virus, 949 Operational taxonomic units, 253 Ophthalmia, gonorrheal, 468 Opisthorcis felineus, 798 O. tenuicollis, 798 O. viverrini, 798 Opsonic index, 382 Opsonins, 381-384 activity of, 383 Orchitis, mumps and, 896 Oriental sore, 783 Ornithine, 152 Ornithodorus hermsi, 749, 750 O. moubata, 749
O. parkeri, 749, 838 O. rudis, 749 O. talaje, 749 O. turicata, 749 Ornithosis, 846, 850-853 chemotherapy of, 849 toxin of, 847 Oroya fever, 603 Orthohydroxydiphenyl, 188 Oryctolagus cuniculus, 876 Osteomyelitis, 507 O'Syl, 188 OT, 673 Otitis media, streptococcal, 445 Otto, 404 Ouchterlony double diffusion technique, 366 Oudin gel diffusion technique, 365 Oven, sterilizing, 13 Oxalacetic acid, carboxylation of pyruvic acid to, 134 Oxalosuccinic decarboxylase, 129 Oxidase(s), 109 cytochrome, 110 Oxidation, aerobic, 106 anaerobic, 106 biological, energy and, 112-114 mechanism of, 107 Oxidizing agents, 186 Oxygen, chemical absorption of, 23

molecular, bacteria and, 111-112

Ozone, water purification by, 319

Oxygenases, 110

Oxytetracycline, 208

Pandemic disease, 293 Pantetheine, 171 Panton-Valentine leucocidin, 419 Pantothenic acid, bacteria and, 171 PAP, 609 Papillomatosis, rabbit, 875 infectious, 877-878 malignancy of, 878 Papovaviruses, 74, 877, 879 Pappataci fever, 965 Paracoccidioides brasiliensis, 689, 729 Paracoccidioidomycosis, 729 Paracolon bacilli, 489-490 Arizona group, 489 Bethesda-Ballerup group, 489 Providence group, 489 Paragonimiasis, 794 diagnosis of, 796 epidemiology and control, 796 Paragonimus westermani, 793 life cycle of, 793 Parainfluenza viruses, 893-895 Parainfluenza 1, 893 Parainfluenza 2, 894 Parainfluenza 3, 894 Paralysis, infantile, 914. See also Poliomyelitis. infectious bulbar, 870 Parashiga bacilli, 516 Parasites, malarial, 783 Parasite populations, host populations and, interaction, 300-303 Parasitology, medical, 769-821 Paratyphoid fever, 505-506 diagnosis of, 502 differential, 505 Paratyphoid gastroenteritis, 505 Paratyphus N2, 506 Paravariola, 862 Paronychia, chronic, 716 Parotitis, epidemic. See Mumps. Parrot fever. See Psittacosis. Particle, virus, structure of, 67 Particulates, submicroscopic, 60 Paschen bodies, 67, 860 Passive cutaneous anaphylaxis, 407 Pasteur, 3, 5, 8, 597, 631, 687, 903, 907 Pasteurella, 557-570 Pasteurella avicida, 557 Past. aviseptica, 557 Past. bollingeri, 557 Past. boviseptica, 557 Past. cuniculicida, 557 Past. hemolytica, 558 Past. leptiseptica, 557 Past. mastitidis, 558 Past. multocida, 557, 558 Past. muricida, 557 Past. muriseptica, 304, 557, 598 Past. pestis, 558-566. See also Plague. bacteriological diagnosis, 564 morphology and staining, 559 physiology of, 560 toxin of, 560 Past. pestis antiqua, 560 Past. pestis mediavalis, 560 Past. pestis orientalis, 560 Past. pseudotuberculosis, 566 Past. septica, 557 Past. suilla, 557 Past. suiseptica, 557 Past. tularensis, 36, 37, 64, 567-570, 605, 825. See also Tularemia. morphology and staining, 567 physiology of, 567 Pasteurellosis, 304

Pancreatitis, mumps and, 896

Pasteurization of milk, 327 Patch test, 674	6-Phosphogluconic acid, formation of, 120	Pleuropneumonia-like organisms, 605-610. See also Mycoplasma.
Paternity, blood group antigens and,	Phospholipids, 138	Pneumococcemia, 457
340 Paul-Bunnell test, 873	synthesis of, 140 Phosphoribulokinase, 132	Pneumococci, 453–461 carriers of, 458
PCA, 407	Phosphorolysis, of sugar, 116	chemotherapy for, 460
Pediculus vestimenti, 749, 835	Phosphorylcholine, 139	classification of, 455 immunity to, 461
Pediococcus cerevisiae, 172 Pedroso, 717	Photophosphorylation, 134 Phthiocerol, 666	infections of, bacteriological diag-
Penicillin, 204, 206	Phthiocol, 666	nosis of, 460
staphylococcal resistance to, 421	Phthoic acid, 666 Phycomycetes, 701	morphology and staining, 453 pathogenicity, for lower animals,
synthetic, 206 Penicillinase, 235	Phycomycosis, subcutaneous, 720–721	460
Penicilliosis, 735, 738	Phylogeny, microbial, 247-249	for man, 457
Penicillium, 701	Physical agents affecting bacteria, 177-	physiology of, 454 resistance to, 458
Penicillium chrysogenum, 206 P. glaucum, 311	184 Physiology, biochemical, fermentation	serological typing of, 456
P. marneffei, 738	and, 4-5	toxins of, 455
P. notatum, 206	Phytomonas polycolor, 397	types of, 456 pathogenicity of, 458
Pentoses, 159 breakdown of, 117–125	Phytotoxins, 266 Picornaviruses, 74, 913–931	transformation, 457
Pentose phosphate pathway, 121	Piedra, 704	variation in, 457
Peptides, synthesis of, 157	Piedraia hortae, 701, 704	Pneumocystis carinii, 729, 793 Pneumoencephalitis, avian, 897
Periodontal disease, 593 Perleche, 716	Pigeon pox, 866 Pigmentation of bacteria, 63	Pneumonia, bronchial, Ascaris lumbri-
Permeases, 53	Pink-eye, 582. See also Conjunctivitis.	coides and, 811
Perognathus baileyi, 727	Pike, red sore of, 492 Pinta, 751, 759–760	epidemic hiberno-vernal, 842 brooder, 736
P. intermedius, 727 P. penicillatus, 727	Pinworm, human, 809	giant cell, 900
Peronychia, 738	epidemiology and control of, 810	influenza and, 892
Peroxidases, 111	infection with, 809	lobar, 457 pneumococci causing, 456
Perroncito, 812 Pertussis. See Whooping cough and	Pipettes, sterilization of, 13 von Pirquet test, 674	of swine, 852
Hemophilus pertussis.	Pithomyces chartarium, 739	plague, 562
Perty, 2	Pityriasis versicolor, 703	plasma-cell, 793 pneumococcal, 457
Petragnani's medium, 18, 665 Petri dishes, sterilization of, 13	Pityrosporum orbiculare, 703 Pixuna viruses, 943	epidemiology of, 459
Pfeiffer, 376	Plague, 558-566. See also Pasteurella	primary atypical, 609, 852
Pfeiffer phenomenon, 376	pestis.	staphylococcal, 424 streptococcal, 440, 445
Pfeiffer's bacillus, 578-581. See also Hemophilus influenzae.	bacteriological diagnosis of, 564 bubonic, 562	virus, 852
Phage, protein of, 71	chemotherapy for, 565	Pneumonitis, 846, 850, 852
Phage typing of staphylococci, 417	cutaneous, 562	pig, 811 toxin of, 847
Phagocytic index, 382 Phagocytosis, factors in, 382	epidemiology of, 563 fowl, 891, 895	Poisoning, food, foodborne infection
immunity and, 389	immunity to, 566	and, 328–333
process of, 382	immunization against, 566	mushroom, 738 Poliomyelitis, acute anterior, 914. See
Phalloidin, 739 Pharyngitis, 937	pathogenicity for man, 568 pneumonic, 562	also Polioviruses.
Pharyngoconjunctival fever, 937	swine, 557	bulbar, 916
Phenol coefficient 195	sylvatic, 563	epidemiology of, 917 experimental infections with, 919
Phenol coefficient, 195 Phenomenon. See under specific name,	vaccine for, 560 Plant viruses, 76–78	immunity to, 919
as Arthus phenomenon.	Plasma membrane, cell wall and, 49-54	nonparalytic, 916
Phenylalanine, biosynthesis of, 153	osmotic regulation and, 52	paralytic, 916
Phialophora verrucosa, 717 Phlebotomus argentipes, 782	Plasmin, 273 Plasminogen, 273	pathogenesis of, 916 predisposing factors, 917
P. caucasicus, 783	Plasmodium berghei, 791	prophylaxis of, 919
P. chinensis, 783 P. major, 783	P. brasilianum, 791 P. cathemerium, 791	vaccines for, 920 Poliomyelitis suum, 921
P. noguchii, 605	P. cynomolgi, 791	Polioviruses, 913, 914-921. See also
P. papatasii, 783, 965, 966	P. falciparum, 786, 787, 788, 789, 790	Poliomyelitis.
P. perniciosus, 783 P. sergenti, 783	P. gallinaceum, 282, 791	antigenic types of, 914
P. verrucarum, 605	P. knowlesi, 791 P. lophurae, 791	pathogenicity for man, 916 replication of, 96
Phlebotomus fever, 965	P. malariae, 786, 787	tissue culture of, 915
Pholiota autumnalis, 739	P. ovale, 787	variation in, 915
Phosphatase test for milk, 327 Phosphate-buffered saline, 27	P. velictum, 791 P. vivax, 783, 784, 786, 787, 788, 789.	Poll evil, 551 Pollen, allergy to, 409, 410
Phosphatidase A, 138	See also Malaria.	Pollender, 6, 614
Phosphatidase B, 139	life cycle of, 783	Polynucleotide phosphorylase, 165
Phosphatidase D, 139 Phosphatides, 138	Plates, culture, preparation of, 21 streak, 22	Polyoma, 879 Polyplax spinulosa, 835
Phosphatidylcholine, 138	Platyhelminthes, 793–821	Polyribosomes, 166
Phosphatidylethanolamine, 138, 140	von Plenciz, 1	Polysaccharides, 115, 344
Phosphatidylserine, 138, 140 Phosphodiesterases, 159	Pleurodynia, epidemic, 923 Pleuropneumonia, bovine, 605, 608	Polysomes, 166 Pomona fever, 762, 765
Phosphofructokinase, 129	canine, 608	Popper, 914
		그 그리다 화장을 하지만 전 시간을 하시었습니다.

Population, bacterial, growth of, 82-86	Pseudomonas (Continued) Ps. nonliquefaciens, 589	Rabies (Continued) pathogenicity, for lower animals, 905
host, 298-300 parasite and, interaction of, 300-	Ps. pseudomallei, 573	for man, 904, 908
303	Ps. pyocyanea. See Ps. aeruginosa. Ps. reptilovorus, 589	prophylaxis of, 909 vaccine for, 909
microbial, 297–298 Pouchet, 3	Ps. saccharophila, 116	Race, resistance and, 279
Poultry plague, pseudo, 897	Ps. septica, 589	Rachmat infection, 765
Powassan virus, 963 Pox disease, pathogenesis of, 861	Ps. syncyanea, 589 Pseudopodia, 771	Radesyge, 751 Radiation, antibody formation and, 393
Poxviruses, 72, 859–867	Pseudorabies, 867, 870	bacteria and, 182
antigenicity of, 860 chemical composition of, 860	Psittacosis, 850–853 epidemiology of, 853	damage from, 183 ionizing, bacteria and, 183
growth of, 860	hypersensitivity in, 852	ultraviolet, bacteria and, 182
morphology of, 859	immunity to, 852	in water purification, 319
of lower animals, 866 replication of, 96	laboratory diagnosis of, 853 morphology and staining, 846	Radiation-pasteurization, 183 Ramibacterium, 600
PPD, 673	pathogenicity, for lower animals, 851	Ramibacterium ramosum, 602
PPLO, 605-610. See also Mycoplasma. Prausnitz-Küstner reaction, 411	for man, 850 serodiagnosis of, 852	Ramon flocculation, 375 Ramon flocculation test of tetanus anti-
Precipitins, 364–367	toxin of, 847	toxin, 629
Precipitin reaction, 30 cross-reactions to, 367	Psittacosis-lymphogranuloma venereum organisms, 66–67	Rat-bite fever, 602–603, 765–766 Rat virus, 502
in gels, 365	chemotherapy of, 849	Ratin, 502
quantitative, 365	experimental infections with, 849	Rattus alexandrinus, 563
with haptenes, 367 Precipitin titration, protocol for, 31	Psychophiles, 178 Puerperal fever, 450	R. decumanus, 765 R. exulans, 563
Precipitinogen, 364	Pulex irritans, 563	R. flavipectus yunnanensis, 841
Preisz-Nocard bacillus, 661 Presbytis entellus, 963	Purified protein derivative (PPD), 673 Purine(s), 158	R. norvegicus, 563, 564, 765 R. rattus, 563, 564
Prigge's toxin, 635	bacteria and, 175	Rayer, 6, 614
Procercoid, 807	biosynthesis of, regulation of, 163	Reaction. See also under specific name. lytic, nature of, 379
Proline, biosynthesis of, 152 Prontosil, 197	metabolism of, 161–163 synthesis of, 160–161	phosphoroclastic, 137
Properdin, 288	Pustule, malignant, 618	serological, 364–385
Prophage, 91 Propionibacterium, 594	Pyridine carboxylic acid compounds, 199	transformation, 242 vaccinoid, 865
Propionic acid, carboxylation to suc-	Pyridinoproteins, 108	Reagents, titration of, 33
cinic acid, 135 fermentation of, 136	Pyridoxamine phosphate, 146 Pyrimidine(s), 158, 159	Reagin, 408 Receptor-destroying enzyme, 203, 886
Propionic acid bacteria, 594	bacteria and, 175	Recombination, conjugation and, 238-
Protection tests, 265	biosynthesis of, regulation of, 163 metabolism of, 161–163	240 viral, 239
Protein(s), acyl carrier, 139 breakdown of, 142–143	synthesis of, 160–161	Recon, 238
metabolism of, 142–157	Pyocyanin, 590	Red fever of the Congo, 836
phage, 71 synthesis of, 163–167	Pyruvic acid, breakdown of, 125–128 carboxylation to oxalacetic acid,	Redi, 3 Reductases, cytochrome, 109
antibiotics and, 209	134	Reed, 7
viral, 68 Proteus, 490–492	Pyruvic oxidase, 129 reactions of, 126	Reed and Frost binomial expansion, 301 Reiter, 763
antigenic structure of, 492		Relapsing fever, diagnosis of, 749
morphology and staining, 490 OX-2, 843		epidemiology of, 749 European, 749
OX-19, 831, 843	Q fever, 826, 827, 828, 841-842	immunity to, 748
OX-K, 831, 843	Quaranfil virus, 960	spirochetes of, 745–750
pathogenicity of, 492 physiology of, 491	Quarantine, in disease control, 307 Quarter evil, 641	Remlinger, 903 Renguera, 907
Proteus hydrophilus, 492	Quellung reaction, 457	Reoviruses, 927
Pr. inconstans, 490 Pr. mirabilis, 490, 491, 492, 831		Replication of animal viruses, 92–99 of bacteriophage, 87–92
Pr. morgani, 491, 492		of herpes virus, 96
Pr. rettgeri, 491, 492 Pr. vulgaris, 142, 143, 144, 324, 490,	R antigen, 436 R colony, 221	of influenza virus, 94 of poliovirus, 96
491, 492, 830, 831	R-factor, 233	of pox viruses, 96
Pr. zenkeri, 490 Protoplasts, bacterial, 53	Rabaul bacilli, 519	of viruses, 86–101
Protothecus zopfii, 717	Rabbit, anaphylactic shock in, 405 myxoma in, 874	characteristics, 87 cycles of, 226
Protozoa, 770–793	papillomatosis of, 875	Resistance, age and, 280
Provirus, 91 von Prowazek, 7, 825	infectious, 877–878 malignancy of, 878	animals and, 278 climate and, 283
Prozone phenomenon, 359	snuffles of, 557	conjunctiva and, 285
Pseudodysentery bacillus, 519 Pseudomonas, 589-590	Rabia parisiante, 907 Rabies, 903–909	drug, gonococcal, 466 staphylococcal, 421
morphology and staining, 589	attenuation of virus, 907	streptococcal, 421
pathogenicity of, 590 physiology of, 589	immunity to, 908	factors in, extrinsic, 300
Pseudomonas aeruginosa, 142, 143, 204,	in bats, 906 in dogs, 905	intrinsic, 299 organ, 287
272, 397, 571, 573, 589–590, 831	laboratory diagnosis of, 908	tissue, 287
Ps. fluorescens, 118, 589	morphology of, 903	fatigue and, 284

Resistance (Continued)	Rickettsia (Continued)	Sabethes choropterus, 960
genital tract and, 286	R. orientalis, 830, 840	Sabin vaccine, 920
hereditary control of, 279	R. pediculi, 842 R. prowazeki, 492, 825, 829, 834, 836,	Sabouraud, 705 Saccharomyces cerevisiae, 713, 735
hormones and, 287	837, 843	S. ellipsoideus, 713
host, 277–289 inherited, 278	R. prowazeki var. mooseri, 834	St. Louis encephalitis, 949-951
intestinal tract and, 286	R. prowazeki var. prowazeki, 834	Salinem infection, 765
microbial, to chemotherapeutic	R. quintana, 834, 842	Saliva, lactobacilli and, 594
agents, 231–236	R. rickettsii, 834, 838, 839, 840	Salivary gland disease, 873-874
mouth and, 286	R. sennetsui, 840, 873	viruses of, 867
nasopharynx and, 285	R. sibericus, 839	Salk vaccine, 920
nutrition and, 283	R. tsutsugamushi, 834, 840	Salmon, 8
race and, 278-279	R. typhi, 834, 836, 837, 843	Salmonella, 495-513
respiratory tract and, 285	Rickettsial disease, laboratory diag-	antigens of, 496
season and, 283	nosis of, 843–844	classification of, 498
sex and, 282	Rickettsialpox, 839	differentiation of, immunological, 496
skin and, 285	Rieckenberg reaction, 371	dissociation of, 500
species, 278	Riff Valley fever, 966–967	ecology of, 501
stomach and, 286	Rinderpest, 899	gastroenteritis from, 504-505
Resistance factor, 233	Ring test, 365	infections from, 503–513
Respiration, bacterial, 106-114	Ringworm, 708	bacteriological diagnosis of, 502
Respiratory enzymes, 106	black-dot, 707	morphology and staining, 495
in bacteria, 107–111	Ringer's solution, 186	pathogenicity for lower animals, 502
Respiratory tract, resistance and, 285	Rio bacilli, 519	physiology of, 495 toxins of, 496
upper, streptococcal infection of, 444-445	Rio Bravo virus, 949 Ristella melaninogenica, 602	typing of, 498
	RNA, 60, 158	phage, 500
Respiratory viruses, 913 syncytial, 895	in bacteria, 57	variations in, 499
Reynolds-Braude phenomenon, 714	messenger, 60, 61, 165	form, 499
Rheumatic fever, 447–448	ribosomal, 166	induced, 500
heredity and, 448	synthesis of, 165	O antigen, 499
penicillin and, 448	transfer, 60, 61, 166	phase, 499
Rheumatism, desert, 726	mRNA, 60, 61, 165	types of, 499
gonorrheal, 468	rRNA, 60, 166	VW, 499
Rhinosporidiosis, 728	tRNA, 60, 61, 166	Salmonella abortus equi, 503
Rhinosporidium seeberi, 689, 728	da Rocha Lima, 7, 825	Sal. abortus ovis, 503
Rhinoviruses, 913, 929	Rocky Mountain spotted fever, 838	Sal. aertrycke, 331, 504
immunity to, 930	Rolling disease of mice, 609, 922	Sal. ballerup, 390, 489
pathogenicity for man, 929	Römer reaction, 375	Sal. barielly, 506
Rhipicephalus appendiculatus, 839	Römer titration of diphtheria antitoxin,	Sal. cholerae-suis, 501, 504, 505
R. sanguineus, 838, 839	_ 29	Sal. cholerae-suis var. Kunzendorf,
Rhizopus, 701	Rosenau, 404	501, 505
Rhodnius prolixus, 780, 781	Rosenbach, 597	Sal. daressalaam, 495
Rhodospirillum rubrum, 132	Ross, 783	Sal. eastbourne, 495, 506
Rhodotorula flava, 713	Ross malaria equations, 301, 303	Sal. enteritidis, 278, 304, 331, 332,
R. glutinis, 713 Riboflavin, bacteria and, 170	Rotlauf bacillus, 598	495, 499, 501, 502, 504, 505 Sal. enteritidis var. Moscow, 506
Ribonucleases, 159	Roundworm, 810 Rous, 880	Sal. gallinarum, 279, 483, 495, 501
Ribonucleic acid. See RNA.	Roux, 6, 649, 752	503
Ribosomes, 60, 166	Rubarth's disease, 934	Sal. hartford, 506
Richet, 404	Rubella, 901–903	Sal. hirschfeldii, 506
Ricketts, 7, 825, 838	immunity to, 902	Sal. montevideo, 499, 501
Rickettsia(e), 825-844	maternal, fetus malformation and,	Sal. newington, 501
bacteria and, 825	902	Sal. newport, 499, 501, 505
chemical composition of, 826	pathogenicity for man, 902	Sal. oranienburg, 506
chemotherapy for, 829	Rubeola, 898–901	Sal. panama, 495, 499, 501, 506
classification of, 831	Ruiz Castaneda test, 554	Sal. paratyphi, 505
cultivation of, 827	Rural typhus, 840	Sal. paratyphi A, 497, 501, 503, 504
experimental infections with, 829	Russian autumnal encephalitis, 961	505
immunity to, 830	Russian endemic encephalitis, 960-962	Sal. paratyphi B, 500, 501, 502, 503
morphology of, 64-66, 826	Russian Far East encephalitis, 960-962	504, 506
pathogenicity of, 828	Russian forest-spring encephalitis, 960-	Sal. paratyphi C, 495, 497, 503, 505
physiology of, 827 propagation of, 25–29	962	506 Sal mullaman 270 405 501 502
resistance of, 828	Russian spring-summer encephalitis, 960-962	Sal. pullorum, 279, 495, 501, 503 Sal. saint paul, 506
specific serological reactions of, 831	epidemiology of, 961	
staining of, 826	immunity to, 961	Sal. schottmülleri, 506 Sal. sendai, 506
toxin of, 269, 828	pathogenicity for man, 961	Sal. senftenberg, 500
viruses and, 825	Russian tickborne encephalitis, 960–962	Sal. simskuny, 500
Rickettsia akamushi, 840	Russian vernal encephalitis, 960–962	Sal. thompson, 505
R. akari, 834, 840	Russula emetica, 738, 739	Sal. typhi, 174, 495, 497, 499, 501
R. australis, 834, 839	Rye, ergot of, 739	502, 503, 504, 507, 508
R. burnetii, 841, 852		Sal. typhimurium, 331, 332, 495, 501
R. conori, 834, 839		502, 504, 505, 506, 850
R. diaporica, 841		Sal. typhimurium (aertrycke), 304
R. mooseri, 834	S antigen, 860, 888, 896	Sal. typhi-suis, 505
R. muricola, 834	S colony, 221	Sal. týphi-suis var. Voldagsen, 505
R. nipponica, 840	SA virus, 894	Salts, bacteria and, 185

	^^		
	Salt solutions, for tissue culture, 27	Sheep (Continued)	Sonne-Duyal bacillus, 521
	San Joaquin fever, 726	enterotoxemia of, 634	Soper binomial expansion, 301
	Sandfly fever, 965–966	facial eczema of, 739	Sore throat, septic, 434, 445
	Sanitation, in disease control, 307 São Paulo typhus, 838	Shellfish, disease and, 332 Shiga, 517	streptococcal, 440, 445 Sororoca virus, 964
	Sarcocystis, 792	Shiga bacillus, 516	South African tick fever, 839
	Sarcocystis lindemanni, 792	toxicity of, 517	Spallanzani, 3
	Sarcodina, 771–775	Shiga-Kruse bacillus, 517	Sparganosis, 808
	Sarcoma(s), fowl, 880, 881	Shigella alkalescens, 517	Sparganum, 807
	Rous, 880	Sh. ambigua, 518 Sh. arabinotarda, 517, 518	Sparganum proliferum, 808 Species of bacteria, 256
	Satranin stain, 19 Scarlatinal toxin, 433	Sh. boydii, 517, 520–521, 525	Specific soluble substances (sss), 344
	Scarlet fever, 434, 445–447	Sh. ceylonensis A, 517	Sphaerophorus, 600
	antitoxin for, 446	Sh. dispar, 517	Spirillum cholerae, 530
	erythrogenic toxin of, 446	Sh. dysenteriae, 517-518, 527	S. cholerae asiaticae (Koch), 530
	penicillin and, 446	type 1, 517 type 2, 518	S. minus, 766
	prophylactic inoculation for, 446 Schereschewsky, 751	Sh. etousae, 520	S. morsus muris, 765 Spirochaeta grippotyphosa, 764
	Schick test, 410, 657	Sh. flexneri, 239, 517, 518-520, 523,	S. icterogenes, 763
	Schistosoma bovis, 802	524, 525, 527, 528	S. morsus muris, 766
	S. haematobium, 800, 801, 802	endotoxin of, 520	S. pseudoicterogenes, 765
	S. intercalatum, 802	types of, 519	Spirochetes, 745–766
	S. japonicum, 800, 801, 802 S. mansoni, 799, 800, 801, 802	Sh. paradysenteriae, 519 Sh. schmitzi, 518	Fontana stain for, 20 of mouth, 750
	S. spindale, 802	Sh. shigae, 517	of relapsing fevers, 745–750
	Schistosomatium douthitti, 802	Sh. sonnei, 517, 521, 522, 525, 527,	Spirochetosis, 748
	Schistosomes, 798	528	Spirocolon, 751
	immunity to, 801	Sh. tieté, 521	Spitz, 574
	life cycle of, 799 Schistosomiasis, 800	Sh. wakefield, 518 Shingles, 871–872	Spleen, antibody formation and, 394, 396
	carcinoma and, 800	Shock, allergic, 411	Splendore, 791
	diagnosis of, 801	anaphylactic, 404	Splenic fever, 617
	epidemiology and control, 801	in dog, 405	Spondweni virus, 949
	Schizont, 784	in guinea pig, 405	Spontaneous generation, 3-4
	Schlomefoher 764	in isolated tissue, 406	Spore(s), bacterial, 54
	Schlammfieber, 764 Schmitz bacillus, 516, 518	in rabbit, 405 mechanism of, 406	formation of, 223 factors affecting, 55
	Schmorl's bacillus, 601	antigen-antibody reaction and, 406	germination of, 57
	Schoenlein, 6, 687	antihistamines and, 407	stain for, 21
	Schröder, 3	endotoxin, 269	sterilization and, 56
	Schüffner's dots, 784	histamine and, 406	Sporidesmin, 739
	Schultz-Charlton blanching phenomenon, 446	Shock organ, 409 Shop typhus, 836	Sporidesmium bakeri, 739 Sporocyst, 794
	Schultz-Dale reaction, 406, 407	Sibbens, 751	Sporotrichin, 720
	Schulze, 3	Siberian tick typhus, 839	Sporotrichosis, 718–720
	Schütz, 570	Silverwater virus, 960	epidemiology of, 719, 720
	Schwann, 3, 4 Schwartzman phenomenon, 276, 573	Simba virus, 965 Simian virus 40, 879	immunity to, 720
	Schweinerothauf, 598	Sindbis virus, 948	ocular, 719 Sporotrichum beurmanni, 719
	Scopulariopsis brevicaulis, 738	Sinusitis, streptococcal, 445	S. equi, 719
	Scrub typhus, 840–841	Siti, 757	S. schenckii, 689, 718-720
	Season of year, resistance and, 283	Skin, resistance and, 285	pathogenicity for man, 719
	Sediment test for milk, 326 Seeber, 729	streptococcal infection of, 443 Slare, 2	Sporozoa, 783–791 intestinal, 791
	Segmenter, 784	Sleeping sickness, 943. See also En-	
Ţ	Selenite F broth, 17	cephalitis.	Spotted fever, 470, 827, 829, 838–840
	Semliki Forest virus, 948	African. See African sleeping sickness.	immunization against, 839
	Semliki-Mayaro virus, 948–949	Slime fever, 764	Rocky Mountain, 838
	Semmelweiss, 5 Sendai virus, 893	Slime layer of bacteria. See <i>Capsule(s)</i> . Slipping of bacilli, 41	vaccine for, 839 Spreading factor, 274
	Septicemia, diphtheria bacillus, 655	Smallpox. See also Variola.	Stachybotrys alterans, 739
	hemorrhagic, 557	black, 862	Stain. See also under specific names.
	mouse, 597	chemotherapy of, 863	acid-fast, 20, 48
	Serine, biosynthesis of, 150	immunity to, 863	capsule, 20
	Serratia marcescens, 178, 272, 479 Serum(s), antitoxic, immunization with,	laboratory diagnosis of, 863 malignant, 862	simple, procedure for, 19
	403	pathogenicity for man, 863	spore, 21 Stain solutions, 19
	horse, hypersensitivity to, 408	vaccine for, 865	Staining, immunofluorescent, 372
	Serum neutralization test, 266	Smears, preparation of, 19	of bacteria, 18
	Serum sickness, 408	Smegma bacillus, 683	of capsules, 46
	Sewage, disposal of, methods for, 319	Smith, Theobald, 8, 404	of rickettsia, 826
	microbiology of, 319–320 Sex, resistance and, 282	Snapping of bacilli, 41 Snow, 5, 6	reactions of bacterial cells to, 47–49
	Sexuality, bacterial, 238	Snuffles, rabbit, 557	Standardization of toxin, 29 Staphylocoagulase, 420
	Sheep, black disease of, 638	Sodoku, 602, 765	Staphylococcus(i), 40, 415–427
	contagious agalactia of, 608	Soil, bacteria from, water and, 311	antigenic structure of, 417
	dysentery of, 634	Solutions, salt, for tissue culture, 27	bacteriological diagnosis of, 425
	encephalomyelitis of, 962	Sonne bacillus, 521, 525, 526	carriers of, 423
		- Particular and the control of th	description of the British of Control of the Contro

Staphylococcus(i) (Continued)	Streptococcus(i) (Continued)
chemotherapy of, 426	infection of, of upper respir
classification of, 423	tract, 444-445
coagulase-positive, 422	morphology and staining, 430
cultural characteristics, 416	nutritive requirements, 431
culture mediums for, 416	pathogenicity, for animals, 439
differentiation between, 423	for man, 439
drug resistance in, 421	penicillin and, 442
enterotoxin and, 419	physiology of, 431
immunity to, 425	variations in, 433
immunization against, 426	Streptococcus agalactiae, 323, 435
infections of, prevalence of, 424	Str. anhemolyticus, 435
suppurative, 423	Str. brevis, 430
morphology and staining, 415	Str. epidemicus, 434, 445
nutritive requirements of, 416	Str. equi, 435, 439
pathogenicity of, 423	Str. equisimilis, 439, 445
for animals, 425	Str. erysipelatis, 434
for man, 423	Str. fecalis, 111, 172, 313, 314,
phage typing of, 417	449, 596
physiology of, 416	Str. hemolyticus, 434, 435
toxins of, 418	Str. lacticus, 323
variation in, 421	Str. lactis, 185, 430
virulence of, 422	Str. longus, 430
Staphylococcus albus, 415, 416, 422,	Str. MG, 441
423	Str. mitis, 439
St. aureus, 63, 175, 197, 229, 323, 330,	Str. mucosus, 456
397, 415, 416, 422, 423, 425, 579,	Str. pneumoniae, 453, 455
596, 698	Str. pyogenes, 215, 297, 323,
St. citreus, 415	439, 440, 441, 442, 443, 444
St. epidermidis, 416	446, 448, 449, 450, 453, 456, 4
St. pyogenes var. albus, 416	Str. salivarius, 439, 449
St. pyogenes var. aureus, 415	Str. scarlatinae, 434, 445
Staphylokinase, 421	Str. viridans, 435
Staphylolysin(s), 270, 418	Str. zooepidemicus, 435, 439
Starch, hydrolysis of, 115	Streptodornase, 275
Stenosis, rectal, 854	Streptokinase, 273, 432
Sterilization, 13-14	Streptolysin(s), 270, 432
by heat, 180	Streptolysin O, 432, 440
intermittent, 13	Streptolysin S, 432, 440
spores and, 56	Streptomyces aureofaciens, 208
Stoll diagnosis of hookworm, 813	S. griseus, 207
Stomach, resistance and, 286	S. madurae, 698
Stomatitis, aphthous, 868	S. rimosus, 208
in horses, 866	S. somaliensis, 698
ulcerative, 601, 868	S. venezuelae, 207
Straus reaction, 572	Streptomycin, 207, 210
Street virus, 907	in gonococcal infections, 466
Strickland reaction, 145	Streptothrix necrophorus, 601
Stricture, rectal, 854	S. muris-ratte, 602
Streptobacillus, 41	Streptotrichosis, 697
Streptobacillus moniliformis, 224, 602-	Strong, 518
603, 608, 609, 765	Strong bacillus, 516
Streptococcus(i), 40, 430–450	Strongyloides ratti, 816
anhemolytic, 434	S. stercoralis, 815–816
chemotherapy of, 442	Struck, of sheep, 634
classification of, 434	Subacute bacterial endocarditis
differentiation, immunological, 435	449–450
physiological, 437	Substance(s), reserve, 59
drug resistance in, 433	specific soluble, 344
erythrogenic toxin of, 432	Subtilin, 205
Group A, 435, 436, 437, 438, 440, 445	Succinic acid, carboxylation of
hemolytic, 433	onic acid to, 135
Group B, 435, 437	Succinic dehydrogenase, 129
Group C, 435, 436, 437, 438, 445	Sugars, complex, hydrolysis of, 1
Group D, 435, 436, 437	synthesis of, 116
Group E, 435	phosphorolysis of, 116
hemolysis of, 434	Sugar broths, preparation of, 15
hemolytic, toxicity of, 431	Suipestifer, American, 504
α-hemolytic, 433, 434, 435, 440	Sulfonamide compounds, 200
β -hemolytic, 433, 434, 435, 440, 445,	Sulfones, 198
447.	Surra, 781
hypersensitivity and, 433	SV40 virus, 879
infections of, bacteriological diag-	Swamp fever, 762, 764
nosis of, 443	Swimmer's itch, 802
epidemiology of, 441	Swimming pool(s), water in, sa
immunity to, 441	control of, 317
of skin and subcutaneous tissues,	Swimming pool conjunctivitis, 85
443–444	Swimming pool granuloma, 683

streptococcus(i) (Continued)
infection of, of upper respiratory
tract, 444-445
morphology and staining, 430
nutritive requirements, 431
pathogenicity, for animals, 439
for man, 439
penicillin and, 442
physiology of, 431 variations in, 433
Streptococcus agalactiae, 323, 435, 439
Str. anhemolyticus, 435
Str. brevis, 430
Str. epidemicus, 434, 445
Str. equi, 435, 439
Str. equisimilis, 439, 445
Str. erysipelatis, 434
Str. fecalis, 111, 172, 313, 314, 439,
449, 596
Str. hemolyticus, 434, 435
Str. lacticus, 323
Str. lactis, 185, 430
Str. longus, 430 Str. MG, 441
Str. mitis, 439
Str. mucosus, 456
Str. pneumoniae, 453, 455
Str. pyogenes, 215, 297, 323, 436, 439, 440, 441, 442, 443, 444, 445,
439, 440, 441, 442, 443, 444, 445,
446, 448, 449, 450, 453, 456, 460
Str. salivarius, 439, 449
Str. scarlatinae, 434, 445
Str. viridans, 435
Str. zooepidemicus, 435, 439
Streptodornase, 275 Streptokinase, 273, 432
Streptolysin(s), 270, 432
Streptolysin O, 432, 440
Streptolysin S, 432, 440
Streptomyces aureofaciens, 208
S. griseus, 207
S. madurae, 698
S. rimosus, 208
S. somaliensis, 698
S. venezuelae, 207
Streptomycin, 207, 210
in gonococcal infections, 466
Streptothrix necrophorus, 601 S. muris-ratte, 602
Streptotrichosis, 697
Strong, 518
Strong bacillus, 516
Strongyloides ratti, 816
S. stercoralis, 815-816
S. stercoralis, 815–816 Struck, of sheep, 634
Subacute bacterial endocarditis, 440,
449–450
Substance(s), reserve, 59
specific soluble, 344
Subtilin, 205
Succinic acid, carboxylation of propi-
onic acid to, 135 Succinic dehydrogenase, 129
Sugars, complex, hydrolysis of, 115
synthesis of, 116
phosphorolysis of, 116
Sugar broths, preparation of, 15
Sugar broths, preparation of, 15 Suipestifer, American, 504
Suipestifer, American, 504 Sulfonamide compounds, 200
Suipestifer, American, 504 Sulfonamide compounds, 200 Sulfones, 198
Suipestifer, American, 504 Sulfonamide compounds, 200 Sulfones, 198 Surra, 781
Suipestifer, American, 504 Sulfonamide compounds, 200 Sulfones, 198 Surra, 781 SV40 virus, 879
Suipestifer, American, 504 Sulfonamide compounds, 200 Sulfones, 198 Surra, 781 SV40 virus, 879 Swamp fever, 762, 764
Suipestifer, American, 504 Sulfonamide compounds, 200 Sulfones, 198 Surra, 781 SV40 virus, 879 Swamp fever, 762, 764 Swimmer's itch, 802
Suipestifer, American, 504 Sulfonamide compounds, 200 Sulfones, 198 Surra, 781 SV40 virus, 879 Swamp fever, 762, 764 Swimmer's itch, 802 Swimming pool(s), water in, sanitary
Suipestifer, American, 504 Sulfonamide compounds, 200 Sulfones, 198 Surra, 781 SV40 virus, 879 Swamp fever, 762, 764 Swimmer's itch, 802

Swine, abortion in, 551 brucellosis of, 551 cholera of, 504 dysentery of, 543 encephalomyelitis of, 921 erysipelas of, 597, 598 gastroenteritis of, 931 influenza of, 891 pneumonia of, 852 Swineherd's disease, 761, 764 Sylvilagus brasiliensis, 876 S. floridanus, 568 Synergism, 204 antagonism and, 214 Syphacia obrelata, 810 Syphilis, 752–757 chemotherapy for, 756 congenital, 754 immunity to, 754 in man, 753 incidence of, 753 latent, 754 nonvenereal, 757 primary stage, 753 secondary stage, 754 serodiagnosis of, 755 tertiary stage, 754 tests for, 755 false reactions to, 756 therapy and, 756 yaws and, 759

T antigen, 436 T-coliphages, 70 T-phages, 70 TAB vaccine, 512 Tabardillo, 835 Tacaribe virus, 955 Tadarida mexicana, 907 Taenia pisiformis, 806 T. saginata, 804 T. solium, 803, 805, 806, 807 epidemiology and control of, 804 immunity to, 804 laboratory diagnosis of, 804 life cycle of, 803 T. taeniaeformis, 806 Taeniorrhynchus fuscopennatus, 928 Tahyna virus, 965 Talfan disease, 921 Tapeworm(s), 802-808 beef, 804 dwarf human, 805 fish, 807 pork, 803 Tatera indica, 563 T. lobengulae, 594 Taxonomy, numerical, 253 of microorganisms, 252-258 Teeth, decalcification of, lactobacilli and, 593 Tejera, 781 Tellurite, staphylococci and, 416 Tembusu virus, 949 Temperature(s), bacteria and, 178 extreme low, 181 Tension, carbon dioxide, 22 Tertanolysin, 626 Teschen virus, 921 Test(s), Abortus Bang Ringprobe, 554 ABR, 554 active protection, 384 agglutination, 497 microscopic, 31 slide, 367

Ascoli, in anthrax, 619

Toxin(s) (Continued) Test(s) (Continued) Tetrathionate enrichment broth, 17 biochemical, medians for, 15 Theiler's virus, 921 of Hemophilus pertussis, 583 Thermal death point, 180 of influenza viruses, 887 complement-fixation, protocol for, 35 Thermal death time, 180 of Listeria monocytogenes, 596 coagglutinating complement adsorpof lymphogranuloma venereum, 847 Thermo-agglutination test, 550 tion, 368 Coombs, 368 Dick, 410, 446 Thermophiles, 178 of meningococci, 472 of ornithosis, 847 Thermoprecipitation test, in anthrax, of Pasteurella pestis, 560 Eagle, 755 619 flocculation, 31 Thiamin, bacteria and, 168 of pneumonitis, 847 Kahn, 755 Thiobacillus thiooxidans, 132, 185 of psittacosis, 847 Thioredoxin, 162 protocol for, 31 of rickettsia, 828 Ramon, for tetanus antitoxin, 629 Thiosemicarbazones, 199 of Salmonella, 496 for syphilis, 755 Thogoto virus, 960 of staphylococci, 418 FPA, 756 Frei, 412, 854 Three-day fever, 928, 965 pneumococcal, 455 Threonine, 149 Prigge's, 635 germicidal equivalent concentration, Thrush, 715 rickettsial, 269 Thuillier, 597 scarlatinal, 433 Thumps, 811 standardization of, 29 Greig, 533 hemagglutination-inhibition, 886 Thymus, immunity and, 402 tetanus. See Tetanus toxin. Hinton, 755 lymphocytopoiesis and, 402 viral, 269 Hotis, 323 Tick fever, 963 Toxin-antitoxin, diphtheria, 658 infection prevention, 196 Kline, 755 Tickborne viruses, 960-964 reaction to, 373 Tiedemann, 816 Toxocara canis, 811 Kolmer, 755 Timothy bacillus, 683 Toxocara cati, 811 KRP, 755 Tinea nigra, 703 Toxoid, 268 mallein, 572 T. versicolor, 703 diphtheria, 658 Tissue(s), host cells and, 98 Mantoux, 674 tetanus, 630 Toxoplasma gondii, 791 life cycle of, 791 methyl red, 484 incompatibility of, 340 Moloney, 658 isolated, anaphylactic shock in, 406 Toxoplasmosis, 792 TPCF test, 755 TPCP test, 755 neutralization, 266, 385 microbial toxins and, 277 passive protection, 385 preparation for culture, 28 patch, 674 subcutaneous, streptococcal infection TPI test, 755 Paul-Bunnell, 873 of, 443 Tissue culture, 27-29 phosphatase, for milk, 327 TPIA reaction, 372 von Pirquet, 674 monkey kidney, 28 Trachoma, 846, 855-856 protection, 265 of animal viruses, 93 Transaldolase, 122 Titration, agglutinin, protocol for, 32 ring, 365 Transaminases, 145 Ruiz Castaneda, 554 antigen, protocol for, 34 Transamination, 145 Transduction, 240–243 Transfection, 240–243 Shick, 410, 657 complement, protocol for, 34 sediment, for milk, 326 hemolysin, protocol for, 33 sensitivity, of chemotherapeutic of diphtheria antitoxin, 29 Transfer factor, 167 drugs, 211 of reagents, 33 Transformation, 241-243 serum neutralization, 266 precipitin, protocol for, 31 factors in, 242 thermo-agglutination, 550 Tobacco mosaic virus, 76 of pneumococcal types, 457 thermoprecipitation, in anthrax, 619 Tobia fever, 838 reaction in, 242 TPCF, 755 TPCP, 755 TPI, 755 Tokushima fever, 873 Transglycosidation, 116 Tolerance, acquired, 342 Transketolase, 122 Tongue, wooden, 574 Translation, 166 use dilution, 196 Tonsillitis, streptococcal, 445 Transcription, 165 Traum, 547 Trench fever, 842 VDRL, 755 Torula histolytica, 735 virulence, for diphtheria, 656 Torulopsis glabrata, 735 in vitro, 366 Vollmer, 674 T. pintolopesii, 735 Trench mouth, 601, 750 Trematoda, 793-802 Treponema, 750-760 Torulopsosis, 735 Wassermann, 380, 755 Toulon typhus, 836 Widal, 511 cultivation of, 751 Toxicity, host origin of, 275-277 Tetanospasmin, 626 of diphtheria bacillus, 652 pathogenicity for animals, 752 Test tubes, sterilization of, 13 Toxicity index, 197 Treponema carateum, 750, 752, 759-Tetanus, 623, 624-630 Toxicosis, Stachybotrys, 739 760 chemotherapy of, 630 Toxin(s), cholerigenic, 534 T. cuniculi, 750 immunity to, 628 crystalline botulinum type A, 266 T. herrejoni, 759 T. macrodentium, 750 T. microdentium, 750, 752 immunization against, active, 629 Dick, 433 passive, 629 erythrogenic, of scarlet fever, 446 in drug addicts, 628 T. pallidum, 746, 750, 751, 752-757, streptococcal, 432 in lower animals, 628 microbial, 266-277 758, 759 in man, 627 tissues and, 277 immune adherence reaction, 372 in newborn, 628 T. pertenue, 746, 750, 752, 757–759 T. recurrentis, 746, 747 murine, virulence of, 561 pathogenicity of, 627 of Bacillus anthracis, 616 postoperative, 628 of Brucella, 549 T. vincentii, 750 surgical, 628 of cholera vibrio, 533 T. zuelzerae, 752 Tetanus antitoxin, 628 of Clostridium botulinum, 643 Treponematoses, 750-760 prophylactic use, 629 of Clostridium histolyticum, 639 Triatoma megista, 779, 780 standardization of, 628 of Clostridium novyi, 638 of Clostridium septicum, 632 Tribec virus, 960 therapeutic use, 629 Tricarboxylic acid cycle, 128-131 Tetanus neonatorum, 628 of Clostridium welchii, 634 as source of intermediates, 129 of coliform bacilli, 484 Tetanus toxin, 267, 626 utilization of energy from, 129 dissemination in body, 626 of diphtheria bacillus, 651 Trichina, 816 Tetrachlorophene, 188 of gonococci, 466 Trichinella spiralis, 810, 816-818 Tetracyclines, 208, 210 of Hemophilus influenzae, 580 Trichinosis, 816

Tuberculosis meningitis, 669 Trichomonas foetus, 776 Ulceration, Buruli, 683 Tuberculostats, antibacterial activity of, Ulcus interdigitale, 697 T. gallinae, 776 T. hominis, 775 672 Umbre virus, 967 T. tenax, 776 synthetic, 671 Una viruses, 943 Tuberculostearic acid, 666
Tularemia, 567-570. See also *Pasteu*-T. vaginalis, 770, 775 Trichomycosis axillaris, 697 Trichophytid, 711 rella tularensis. water standards of, 316 bacteriological diagnosis of, 569 chemotherapy of, 569 Trichophytin, 712 Trichophyton concentricum, 709-710 T. mentagrophytes, 707, 709 epidemiology of, 568 Uruma viruses, 943 glandular, 567 immunity to, 569 T. rubrum, 712 T. schoenleinii, 687, 707, 709, 710 Use dilution tests, 196 Usutu virus, 949 T. tonsurans, 711 immunization against, 570 Uta, 783 insect vectors of, 569 pathogenicity of, 567 T. violaceum, 687 Uveitis, histoplasma, 738 Trichosporon cutaneum, 704 typhoidal, 567 Trichostrongylus orientalis, 814 Trichuris trichiura, 810, 816 ulceroglandular, 567 Triglycerides, synthesis of, 140 vaccine for, 570 Trivittatus virus, 965 waterborne, 569 Trombicula akamushi, 841 Tumor, fungous, 698 V antigen, 561, 888, 896 Tumor viruses, 874-882 T. deliensis, 841 Vaccine(s), anthrax, 620 Tupaia belangeri versurae, 841 T. fletcheri, 841 T. walchi, 841 Turlock virus, 967 brucellosis, 555 Tronchado, 907 Twort, 7 Trophozoite, ameboid, 784 Twort-d'Herelle phenomenon, 69 cholera, 541 Tyndall, 3 erysipelas, 599 Tropical bubo, 853 Trout, ulcer disease of, 492 Typhoid bacillus, 503 plague, 560 Trudeau medium, 665 Trypanosoma brucei, 781 Typhoid fever, 505-513 carriers of, 508 T. cruzi, 772, 776, 779, 780 cholecystectomy in, 509 pertussis, 585 chemotherapy for, 508 complications of, 507 life cycle of, 779 polyvalent, 402 T. evansi, 781 smallpox, 865 T. gambiense, 776, 777, 778, 779, 781 life cycle of, 777 T. lewisi, 389, 749, 781 T. rangeli, 776, 781 T. rhodesiense, 749, 776, 778 diagnosis of, 502 rabies, 909 endemic, 510 epidemiology of, 509, 510 spotted fever, 839 TAB, 512 foodborne, 510 tularemia, 570 immunity to, 510 typhoid fever, 512 T. vivax, 778 milkborne, 510 mouse, bacillus of, 504 Trypanosomes, 777-781 Weigl, 837 Trypanosomiasis, 749, 777, 778 diagnosis of, 778 passive immunization against, 513 yellow fever, 960 prophylactic inoculation against, 512 epidemiology and control of, 778 signs and symptoms of, 507 generalized, 864 immunity to, 778 waterborne, 510 immunization and, 864 Tryptophan, biosynthesis of, 153 Typhoid Mary, 510 Typhus, 826, 828, 829, 834–838 breakdown of, 144 Valley fever, 726 Tryptophan broth, preparation of, 15 endemic, 834 Tsetse flies, typanosomiasis and, 777 Tsutsugamushi, 829, 830, 840-841 epidemic, 834 in cell structures, 222 louseborne, 835 immunization against, 840 European, 834 immunization against, 837 Tubercle, 669 nature of, 243-247 Tubercle bacillus(i), 663-678 Indian tick, 839 observed, 220-243 chemical composition of, 666 Kenya, 839 cord factor of, 666 latent, 835 strate, 227 Varicella, 871–872 drug resistance of, 667 Manchurian, 836 mite, 840-841 filterable forms of, 667 growth in cell culture, 665 Moscow, 836 infection by, routes of, 668 spread in body, 669 murine, 834, 835–837 North Queensland tick, 839 Smallpox. Variola major, 862 Variola minor, 862 Variolation, 864 life cycles of, 667 rural, 840 São Paulo, 838 scrub, 840-841 morphology and staining, 663 pathogenicity for man, 668 physiology of, 664 shop, 836 VDRL test, 755 Siberian tick, 839 resistance of, 666 Toulon, 836 variation in, 667 Tuberculin, 673 vaccine for, 837 of tularemia, 569 Tyrocidin, 205 reaction to, 411, 674 of WEE, 945 Tuberculin residuum (TR), 673 VEE virus, 947-948 Tyrosine, biosynthesis of, 153 Tuberculosis, 668 Veillon, 633 Tyrothricin, 205 active, immunization against, 675 bacteriological diagnosis of, 670 V. discoides, 477 bone and joint, 669 V. orbiculus, 477 chemotherapy of, 671 V. parvula, 477 epidemiology of, 677 V. reniformis, 477 heredity and, 670 U virus, 894 immunity to, 672 Uganda S virus, 956 Uhlenhuth, 763 mechanism of, 674 pathogenicity for lower animals, 675 Ulcer, chiclero, 783 Verruga peruana, 603 predisposing factors in, 670 laryngeal, 507 Vi antigens, 497

Undulant fever, 551. See also Brucellosis U.S. Public Health Service, drinking Urethritis, mongonococcal, 477, 610,

attenuated, for typhus, 837 bacillary dysentery, 527 poliomyelitis, inactivated, 920 living attenuated, 920 typhus, inactivated, 837 Vaccinia, 860, 861, 862-866 Valine, biosynthesis of, 149 Variation(s), discontinuous, 230 microbial, biochemical, 229-231 genetics and, 218-249 physiological, 227-236 selection of medium to demon-Variola, 860, 861, 862-866. See also experimental infections of, 865 Vectors, insect, control of, 307 of brucellosis, 553 Veillonella alkalescens, 477 V. vulvovaginitidis, 477 Venezuelan equine encephalitis, 947Virus(es) (Continued)

Vibrios, Celebes, 532 El Tor, 530, 532 NAG, 536 Vibrio cholerae, 530-542, 532, 533, 535, 536, 537, 543, 886 V. coli, 543 V. comma, 536 V. comma (Bergey), 530 V. danubicus, 542 V. eltor, 536 V. fetus, 530, 543 V. ghinda, 542 V. jejuni, 543 V. massauah, 542 V. metchnikovii, 530, 543 V. parahaemolyticus, 543 V. phosphorescens, 543 V. piscium, 543 V. proteus, 543 Vibrion septique, 631-633 Villesmin, 663 Vincent's angina, 593, 601, 750 Violacein, 205 Viremia, 861 Virulence, animal passage and, 229 bacterial, loss of, 229 modification of, 229 capsules and, 275 hemolysin and, 271 leucocidins and, 272 measurement of, 263 ED₅₀, 264 microbial, 263–277 mucin and, 265 of staphylococci, 422 Virulence antigen, 497 Virulence test, for diphtheria, 656 in vitro, 366 Virus(es), 7-8. See also under specific designations, as Arbovirus(es), Enterovirus(es), etc. acute laryngotracheobronchitis, 894 adeno-pharyngeal-conjunctival, 935 adenoid degenerative, 935 adsorption of, 87 ALTB, 894 animal. See Animal viruses. APC, 73, 935 auto-interference of, 100 B, 867, 870 bacterial, 69-72 Bunyamwera, 964 bushy stunt, 78 Bwamba fever, 967 CA, 894 Cache Valley, 964 California, 967 And Juneta Lings of the property of the f capsid of, 69 CCA, 895 chemical composition, 68 Chikungunya, 948 classification of, 258 Coe, 923 Columbia SK, 928 common cold, 929-930 Copenhagen, 222, 894 Coxsackie. See Coxsackie virus(es). croup associated, 894 Danysz, 504 duck egg, 904 EA 102, 895 16 Supplies Jan 17 13-64-58/6-3 ECBO, 924 1 s in atus kurenta ni∀ ECDO, 924 ECHO. See ECHO virus(es). ECMO, 924, 928 ECSO, 924

EEE. See EEE.

EMC, 928-929 encephalitis, 943-949 encephalomyocarditis, 928-929 ERC, 929 FA, 921 fixed, 907 HA, 893 HA 2, 894 hemagglutinating, of Japan, 893 unit of, determination of, 888 hepatitis, 913 Ilheus, 957 incomplete, 95 infectious wart, 877 influenza. See Influenza virus(es). insect, 75-76 interference of, 99-101 kind, 100 nature of, 100 Junin, 955 Kumba, 948 M-25, 895 Machupo, 955 Makonde, 948 masked, 100 Mayaro, 948, 949 Mengo, 928 MM, 928 morphology of, 67-78 mosquito-borne, 949-960, 965-967 Mossuril, 965 NDV, 895 newborn pneumonitis, 893 Ntaya, 956 O'nyong-nyong, 949 plant, 76-78 Powassan, 963 pox. See Poxviruses. propagation of, 25-29 protein of, 68 rabbit papilloma, 877-878 rat, 502 replication of, 86-101 characteristics, 87 cycles of, 226 respiratory, 913 syncytial, 895 rickettsia and, 825 SA 894 salivary gland disease, 867 Semliki Forest, 948 Semliki-Mayaro, 948-949 Sendai, 893 Sindbis, 948 street, 907 structure of, 68 symmetry of, 69 Tacaribe, 955 Theiler's, 921 tickborne, 960-964 tobacco mosaic, 76 tumor, 874–882 Turlock, 967 U, 894 Uganda S, 956 Umbre, 967 vacuolating, 879 VEE, 947-948 vegetative, 89 Wad Medani, 960 WEE. See WEE. Wesselsbron, 949 West Nile, 953 Witwatersrand, 960, 965 wyeomyia, 965 xanthematous disease, 859-882 Yale SK, 928

Virus(es) (Continued)
Zika, 957
Virus particle, 67
Visceral larva migrans, 811
Vitamin(s), amino acids and, 175
B complex, bacteria and, 168
B₆, bacteria and, 170
B₁₂, bacteria and, 173
Voges-Proskauer reaction, 136, 485
Vole bacillus, 682
Vollmer test, 674
Vulvovaginitis, gonorrheal, 468

W antigen, 561 Wad Medani virus, 960 Waksman, 207 Warburg's respiratory enzyme, 110 Wart, genital, 877 human virus of, 877 Warthin-Finkleday cells, 900 Wassermann, 9, 755 Wassermann test, 380, 755 leprosy and, 682 Water, analysis of, bacteriological, 313 chemical, 316 bacteria in, factors in, 313 plate counts of, 315 contamination of, by excreta, 312 microbial indicator, 315 deep, 313 drinking, 316 purification of, 317 filtration of, 317 flowing, self-purification of, 319 microbiology of, 310-319 natural, bacteria native to, 311 contamination of, bacterial, 311 purification of, by boiling, 319 by ozone, 319 by ultraviolet irradiation, 319 chemical, 318 mechanical, 317 sanitary quality of, 316 surface, 313 swimming pool, sanitary control of, 317 Water fever, 764 Waterhouse-Friderichsen syndrome. Watsonius watsoni, 798 WEE, 944-946 virus of, immunity to, 944 pathogenicity for man, 944 vectors of, 945 Week, 581 Weichselbaum, 6, 470, 570 Weigert, 6 Weigl vaccine, 837 Weil-Felix reaction, 492, 830, 843 Weil's disease, 763-764 Welch, 633 Wells, water from, 313 Wesselsbron virus, 949 West Nile virus, 953 Westerman, 793 Western equine encephalitis. See WEE. Whipworm, 810 Whitmore's bacillus, 573-574 Whooping cough, 582-586. See also Hemophilus pertussis, bacteriological diagnosis of, 584 chemotherapy of, 584 epidemiology of, 584 immunity to, 585 immunization against, 585 Widal, 9, 517

Wyeomyia melanocephala, 965

Widal test, 511 Wilfarth, 5 Wilsdon types of Clostridium welchii, 634 Winogradsky, 5 Withers, fistula of, 551 Witwatersrand virus, 960, 965 Wöhler, 4 Wolbachia melophagi, 834 Wolhynian fever, 842 Wooden tongue, 574 Woods, 10 Wool sorter's disease, 618 World Health Organization, drinking water standards of, 317 Worm, eye, 820 Guinea, 820 Wounds, infection of, streptococcal, 444 war, complication's of, 623 Wright, 783 Wuchereria bancrofti, 818, 819, 820

life cycle of, 818

Xanthematous diseases, viruses of, 859-882
Xenodiagnosis, in Chagas' disease, 780
Xenopsylla cheopsis, 605, 835
Xerosis, 661

Yale SK virus, 928
Yato-byo, 569

Yato-byo, 569
Yaws, 746, 751, 757-759
Scottish, 751
syphilis and, 759
Yeasts, pathogenic, 712-716
Yellow fever, 957-960
African, 959
Central American, 959
epidemiology of, 958

Yellow fever (Continued)
experimental infections with, 958
immunity to, 960
jungle, 959
pathogenicity for man, 957
prophylaxis of, 960
South American, 959
urban, 958
vaccine for, 960
Yersin, 6, 559, 649
Yolk sac, propagation of microorganisms in, 26

Ziehl-Neelsen stain, procedure for, 20 Ziehl's carbolfuchsin, 19 Zika virus, 957 Zootoxins, 266 Zoster, 871–872 Zuber, 633 Zymosan, 288